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(MIKROBIOLOGIYA)

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MICROBIOLOGY

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DECEMBER PLENARY SESSION OF THE CENTRAL COMMITTEE OF THE CPSU AND TASKS IN MICROBIOLOGY

Translated from Mikrobiologiya Vol. 29, No. 1, pp. 3-7, January-February, 1960

The plenary session of the Central Committee which convened December 22-25, 1959, in Moscow was an important event in the life of the party and Soviet people.

At this session the growth of agriculture in the country was reviewed for 1959, the first year of the Seven Year Plan for developing the national economy of the USSR. This review convincingly showed that the agricultural workers, effectively carrying out the historic resolutions of the Twenty-First Congress of the CPSU and December, 1958, plenary session, have achieved remarkable new progress. Our agriculture is on the proper path and is confidently headed toward a new rise.

A good harvest was gathered in the Soviet Union as a whole, despite the unfavorable climatic conditions in certain regions of the country. Livestock production made substantial progress: production and delivery of meat and milk increased markedly.

In 1959, our country surpassed the United States in per capita production of butter; our gross milk production was greater than in the United States.

Of exceptional importance is the fact that the socialist sector has now become the potent main source of supply of meat and milk to the cities.

The rapid growth in all branches of agriculture is a result of the heroic labor of worker, kolkhoz farmers, and Soviet intellectuals, of the great creative and purposeful work of the party.

The plenary session of the Central Committee of the CPSU, in noting the achievements of socialist agriculture, at the same time disclosed serious shortcomings in a number of republics, territories, and provinces.

Urgent tasks for a further rise in all branches of agriculture were defined and formulated clearly in the plenary session decree.

All the necessary conditions have been created for us to carry out these tasks effectively. Our state provides the kolkhozes and sovkhozes with modern equipment and is constantly concerned with the needs of rural workers. Many thousands of heroes and heroines of labor have appeared in the countryside in recent years, revealing to agriculture such inexhaustible reserves as was difficult even to dream of previously. Many of these outstanding innovators in kolkhoz and sovkhoz projects participated actively in the plenary session of the Central Committee of the CPSU.

"This plenary session," said N. S. Khrushchev in his speech, "is a plenary session to mobilize the reserves of agriculture." The main objective at present is, guided by the experience of front-rank workers, to put into action the inexhaustible reserves of agriculture.

The role of agricultural science in fulfilling the great tasks set before agriculture by the December plenary session of the Central Committee of the CPSU has been greatly increased.

Workers in agricultural research institutions an educational establishments and at experiment statio should exert a maximum effort to promote in every wa a further rise in crop yields, increase in livestock, an livestock products.

Workers in agricultural science should give partic ular attention to rendering greater practical assistanc to kolkhozes and sovkhozes in the matter of increas ing crops and livestock, and should actively aid the in properly solving their problems.

Agricultural science should work out new, progres sive, scientifically substantiated methods and proce dures in agricultural practice and actively promo their widespread adoption.

The first task is, through the combined efforts o scientists in different specialties, to place more rapid ly and fully at the service of production all the achieve ments of agricultural science and vanguard experienc

A most important task of the scientific researc institutions is the further strengthening of ties wit production.

Comrade N. S. Khrushchev, in his speech at the ple nary session, gave a number of valuable indications o how best to achieve the strengthening of ties betwee agricultural science and production. He said, "We mus bring science still closer to production, send more o our capable, gifted young scientists to the Academy o Agricultural Science and to the institutes, so that the may enrich science with their knowledge and hel practical workers engaged in agriculture. Scientifi institutions should conduct a large part of their studie in line with practical problems."

Execution of these instructions and reorganization o the system of aid to kolkhozes and sovkhozes on th part of scientific institutions and experiment station will unquestionable promote a rise in agricultura productivity. This rise in turn will be highly beneficia to agricultural science by promoting sound and faste development.

Successful fulfillment of the tasks facing agricultura science requires of our scientists, as noted in th plenary session decree, a further rise in the level o theoretical research through fuller use of the mos recent achievements in biology, physics, chemistry and related sciences. By expanding such researc further rise in productive power and continuous techni cal progress in agriculture will be assured.

In fulfilling the tasks confronting agricultural scienc as a whole, agricultural microbiology has an importan role to play.

Increasing the yield of all crops is a most importan task for agriculture. Microorganisms are known t play an essential role in transforming matter in the soil and in enriching it with vitamins, growth substances, enzymes, and other physiologically active substances, thus influencing the plant's root nutrition and eventually the yield.

Soil microbiologists, in close collaboration with other experts (soil scientist, agrochemists, agronomists, etc.), should develop and apply to agriculture such methods of treating soil and of preparing, storing, and applying mineral, mineral-organic, and bacterial fertilizers, and such methods of crop rotation, as will make maximal use of the biochemical activity of soil microorganisms and their products to raise the effective fertility.

To fulfill this task successfully, thorough study is necessary of growth characteristics of the microorganisms forming the natural flora in the soil and in the rhizosphere, of the relationships among them and to higher plants in the soil. It is necessary to expand research on the physiology of soil microorganism, and to increase the study of producers of physiologically active substances—plant growth stimulators—and of possibilities for their practical use.

Due attention should be given to studying the role of soil fungi, algae, and fauna in plant nutrition and soil fertility.

Serious attention should also be given studies to determine the nature of the action of bacterial fertilizers and the conditions determining a positive action. Of importance in solving this problem are improvement in the technology of preparing all bacterial products in use, development of methods for producing standard dried products, and standardization of instructions for use.

Agricultural microbiology has an important part to play in solving the problems confronting our livestock production. The further increase in livestock and sharp rise in livestock products planned by the plenary session requires that agricultural workers take measures to establish a stable feed base and to provide livestock with adequate amounts of succulent and protein feed, vitamins, and other nutrients.

Corn is our basic feed crop. The efficient use of this crop affords exceptionally favorable opportunities to increase the feed base and hence the number of livestock. Improving methods for silaging feed is a most important task for agricultural microbiologists in collaboration with other experts. Measures to reduce waste during silaging and to raise quality and decrease cost of fodder are of particular importance.

The method of silaging corn on the ground is of great importance in our present-day large-scale livestock production, since it is the simplest. In this method ways must be found to prevent spoilage of the surface layer of fodder and heat generation within the fodder, result in considerable waste and much loss of food value.

Measures to enrich fodder, particularly from corn, with proteins and nitrogen must be improved. Hence studies on the possibility of adding a mixture of legumes, ammonium salts, and phosphates to fodder, and studies on defining methods to improve the silaging of feed difficult to silage, in particular protein-rich grasses, merit serious consideration.

Due attention should be given to developing methods for utilizing ferments of lactic acid bacteria to improve fodder quality, to selecting the most effective cultures of lactic acid bacteria for this purpose, and to developing technology for mass production of dried fodder ferments.

Food yeasts are an important source for enriching feed with proteins and vitamins. Hence studies should be greatly expanded on further improving the technology of growing food yeasts on cellulose hydrolyzates, sulfide liquor, and other nonfood sources, and on selecting the yeast cultures richest in protein and vitamin contents.

The search for additional sources of vitamin products for livestock needs, by growing microorganisms on various types of nonfood material, is becoming of much practical importance.

The experience gained in recent years by kolkhozes and sovkhozes in the use of certain antibiotics in raising livestock has demonstrated their great effectiveness beyond question. A most important task is further to improve the production technology of tested feed antibiotics, to take measures for providing livestock raisers with standard products, and to devise rational methods for enriching mixed feed with antibiotics. The ever-increasing use of antibiotics in livestock raising urgently demands the working out of scientifically based requirements for feed antibiotic products and the organization of quality control over these products. It is also necessary to search for new antibiotics suitable for use in raising livestock.

Of obvious interest is the study in animals of the gastrointestinal microflora participating in protein synthesis from urea or ammonium salts.

Problems relating to quality improvement of agricultural produce and curtailment of waste are gaining particularly great significance at the present stage.

In our large-scale production each new step in this direction produces an enormous effect. Hence, the problems of improving quality of produce and combating waste should become the object of particular concern to scientific and practical workers, including microbiologists.

More effective measures must be developed to combat waste of agricultural produce caused by fungal plant pathogens, bacteria, viruses, and various crop pests.

Certain antibiotics and microbial preparations have been proposed by Soviet scientists and found to be effective in combating many harmful diseases of crops. The development of production technology and methods for practical use of these products must be accelerated, and the search for new ones expanded.

The struggle for quality produce and waste reduction does not end with harvesting. Of great economic importance are measures designed to improve conservation and methods of treating agricultural produce in order to minimize waste at all stages up to final consumption.

Veterinary microbiologists are confronted with serious tasks in the matter of further improving present methods of combating infectious diseases in domestic animals and of devising effective new ones.

The problem of rational use of agricultural waste products has great practical significance. Microbiological science must render substantial assistance to agriculture in solving this problem.

In certain countries in recent years, anaerobic (methane) fermentation of agricultural waste products has been widely employed. This method serves to solve a number of problems simultaneously including those

of obtaining cheap local power and fertilizers. It may be of importance in certain parts of our country, and study of the question deserves attention.

Today, since the network of scientific research institutions working in the field of agricultural microbiology has become very large, the coordination of their activity becomes of particular significance. This question should be given particular consideration by the Academy of Sciences of the USSR and the V. I. Lenin Academy of Agricultural Sciences.

There can be no doubt that Soviet microbiologists, in close collaboration with agricultural workers, will direct all their efforts and all their creative energy toward fulfilling ahead of schedule the tasks set for them by the Party and Government, that of producing an abundance of agricultural products in the country.

PHOSPHORUS METABOLISM IN ASPERGILLUS NIGER T-1 VARIANT OBTAINED BY ULTRAVIOLET IRRADIATION

I. Cultural and Biochemical Differences Between the Parent and Experimental Strains

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Variations in microorganisms produced by powerful agents have interested many microbiologists. Extensive studies on comparing the morphological and physiological properties in the variants obtained afford abundant data confirming the existence, in a number of cases, of a correlation between morphological and physiological properties in the variants, thus aiding in establishing principles for the selection of highly active microorganism cultures (Imshenetskii, 1951). Radiant energy has great activity and a knowledge of its mechanism of action on microbes is essential to the solution of a number of problems in general biology.

Citric acid formation is one of the steps in the cell metabolism of Aspergillus niger. It would be expected that changes in acid-forming ability of various strains would be accompanied by other differences in their physiology and metabolism as well. This has not been adequately studied. An attempt has been made in the present paper to describe the physiological and biochemical characteristics of an Aspergillus niger mutant obtained by ultraviolet irradiation.

METHODS

Methods of Culturing Organisms.

The studies were made with a parent culture, Aspergillus niger (6/5, and a variant of this organism (T-1) obtained by ultraviolet irradiation (Imshenetskii, Solntseva, and Kuranova, 1959).

Zhuravskii's medium of the following composition was used as the basic medium in the experiments (in g): sucrose—50; NH_4Cl—4; $MgSO_4 \cdot 7H_2O$—0.5; KH_2PO_4—0.5; $ZnSO_4 \cdot 7H_2O$—0.05; $FeSO_4$—0.025; distilled water—1 liter; pH was brought to 4.2 with hydrochloric acid. Sterilization was conducted at 0.5 atm for 30 minutes. Aspergillus niger conidia from parent culture and variant were obtained by Zhuravskii's method of growing cultures on wort agar containing 1.5% NaCl for 4 days at 32 deg and then suspending the conidia in water and seeding them on media. Duration of the experiments was six or seven days. Two days after seeding, the solutions were replaced. The nutrient medium was poured off from under the pellicle, the lower surface of the fungus pellicle twice washed with distilled water; and 25 ml 20% sugar solution containing 0.2% NH_4Cl poured under it. The fungus spent 4 days in the sugar solution. In experiments without replacement, the fungus grew on the same Zhuravskii medium for 6 days. Biochemical studies of the fungus were begun two days after seeding, when the mycelium as a very delicate pellicle covered the surface of the liquid medium.

Fixation and Drying of Material.

The nutrient medium or sugar solution was poured off from under the pellicle; the pellicle was washed repeatedly with distilled water; then the pellicle was shaken twice with cold alcohol, the final concentration being not less than 80 deg. The alcohol-treated pellicles were dried in a desiccator over phosphorus anhydride in a low vacuum at room temperature. The substances extracted by the alcohol were estimated from the residue weight, after evaporation of the alcohol. The material thus obtained was used for further analyses.

Fractionation of Fungal Phosphorus Compounds.

a) Cold Acid-Soluble Fraction. The dried fungus pellicles were thrice extracted with 5% trichloroacetic acid (TCA) by the method of Bajaj and Krishnan (1953) at 0 deg for two hours; the first time for an hour, the second and third times for half an hour with centrifugation. The centrifugates of the three extractions were combined and the precipitate was washed once again with water. Wash waters were combined with the extract. In the extract were determined: 1) Inorganic phosphorus; 2) phosphorus mineralized after 10-min hydrolysis with 1 N H_2SO_4 on a boiling water bath; 3) total phosphorus after combustion with 40% $HClO_4$. Labile phosphorus was calculated from the difference between inorganic phosphorus and phosphorus, determined after 10-min hydrolysis; stable phosphorus was the difference between total phosphorus and phosphorus determined after 10-min hydrolysis.

b) Phospholipids. To the residue remaining after cold 5% TCA extraction, alcohol was added. After mixing, the alcohol was removed by centrifuging and extraction carried out three times on the water bath with 70 deg alcohol-ether mixture 1:3, each time for 20 min. Phospholipids and other phosphorus-containing lipoids were determined from the total phosphorus in the alcohol and alcohol-ether extracts.

c) Protein-bound Phosphorus. Schneider's method 1945, slightly modified, was used to determine various protein-bound phosphorus compounds in the acid-insoluble residue.

For isolation of nucleic acids, the residue remaining after alcohol-ether extraction was heated with 0.5 N perchloric acid in a boiling water bath for 20 minutes.

The residue was removed from the extract by centrifuging and twice washed with 0.5 N perchloric acid. Nonnucleotide phosphorus (i.e., polyphosphates) was calculated from the difference between total phosphorus and total amount of nucleic acids in the $HClO_4$ extract.

The insoluble precipitate remaining after extraction with perchloric acid was treated by combustion with concentrated $HClO_4$ and the residual phosphorus in it determined.

Determination of Nitrogen, Phosphorus, Ash, and Sugar.

Inorganic phosphorus, total phosphorus, and phosphorus in the various fractions was determined after combustion with $HClO_4$ by the Fiske-Subbarow method (as modified by Umbreit, 1949). Nucleic acid phosphorus was determined spectrophotometrically by the method of Spirin (1958). Total nitrogen was determined by the usual Kjeldahl micromethod (Belozerskii, 1951); ash, by the combustion method. Organic acids were determined by titration with 0.1 N NaOH. Residual sugar was determined by the method of Shaffer and Somogyi (1933).

EXPERIMENTAL

Cultural Characteristics of Parent and Experimental Strains.

Preliminary experiments on comparative study of these strains showed that they differed in growth rate, sucrose consumption, and dynamics of citric acid formation.

The results of these studies are shown in Table 1 and Fig. 1. On examining these results it was plain to see that the strains studied differed not only in acid-forming ability, but in a number of other physiological properties as well. Strain T-1 differed from the parent strain 6/5 in its slower growth and correspondingly slower sucrose consumption. Strain T-1 began to produce acid later, but produced more than the parent strain 6/5. Table 1 shows that when these data are calculated per unit of biomass the experimental strain T-1 is highly active, not only in forming

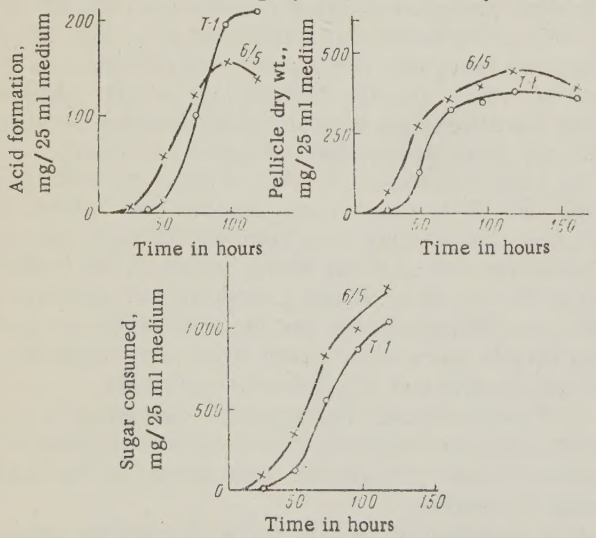

Fig. 1. Biomass production, sucrose consumption, and acid formation in Aspergillus niger parent strain 6/5 and its variant T-1.

acids, but also in assimilating carbohydrate. In comparing the coefficients of biomass synthesis and of acid formation, it may be said that at all growth stages the energy bound by the cells during sucrose cleavage by polyenzyme systems is expended largely for biomass synthesis in the parent strain, and for acid formation in the modified strain T-1.

As seen from Fig. 1, strain T-1 forms about 30% more acids than strain 6/5. Growth rate of strain T-1 is slower than strain 6/5; it lags behind strain 6/5 in biomass production throughout growth, particularly in the early stage. The higher growth rate and consumption of nutrients noted in strain 6/5 produce early pellicle maturation, and hence the phase of acid biosynthesis sets in earlier.

The slower growth found in T-1 results in lengthening the phase of acid formation.

Over-all Chemical Composition and Various Phosphorus Fractions in Pellicles.

It is obvious today that the basis of altered growth processes in microbes obtained as a result of ultraviolet irradiation is an alteration in metabolic processes.

In order to describe more fully the comparative biochemistry of strains T-1 and 6/5, total nitrogen, phosphorus, and ash were determined in the fungal mycelium. The results obtained are given in Table 2. It is clear from these results that the experimental strain T-1 accumulates more inorganic phosphorus and nitrogen salts, especially in early growth stages, than strain 6/5. This indicates that these strains differ not only in acid formation, but in a number of other properties as well.

It is known from the literature that a mutant of the tobacco mosaic virus differed considerably from the parent strain in amino acid composition. Stanley (1947) and Diller et al. (1950), studying an Aspergillus niger mutant obtained by the action of mild x-rays, demonstrated considerable deviations in amount of certain amino acids and nucleic acids between the mutant and parent strains.

The fact that the experimental variant T-1 has a higher total phosphorus content was shown in Table 2. It was of interest to determine what compounds were responsible for the phosphorus accumulation in cells of the variant. For this purpose various forms of phosphorus in both the acid-soluble and acid-insoluble fractions of the fungal mycelium were determined.

Chemical determination of various phosphorus compounds in the experimental variant showed that appreciable phosphorus accumulation occurs. It follows from the data in Table 3 that a higher phosphorus content in variant T-1 is observed in all the fractions studied.

These preliminary results seem to indicate some sort of shift in the phosphorus metabolism of the variant obtained by ultraviolet irradiation.

In conclusion, I take this opportunity to express my deep gratitude to Professor A. A. Imshenetskii and E. L. Ruban for advice and criticism in the course of the work.

SUMMARY

1. An Aspergillus niger variant (T-1) obtained by ultraviolet irradiation is distinguished from the par-

Table 1. Biomass Production, Sucrose Consumption, and Acid Formation during Growth of Aspergillus niger 6/5 and Experimental Strain T-1

Expt. no.	Strain	Age (day)	Mycelium, wt., mg/25 ml medium	Sucrose consumption, %	Acid formation, mg/25 ml fluid	Sucrose consumption per unit of biomass, mg/mg	Acid formation per unit of biomass, mg/mg	Coefficient of biomass, synthesis*	Coefficient of acid formation†
I	6/5	2	79.1	28	105.7	4.4	–	0.23	0.32
		3	210.0	66	125.5	3.9	0.59	0.25	0.15
		4	261.0	84	158.8	4.0	0.61	0.24	0.15
		5	274.1	100	139.6	4.6	0.51	0.22	0.11
	T-1	2	22.7	8	78.7	4.4	–	0.23	0.79
		3	115.4	44	101.7	4.8	0.89	0.21	0.19
		4	182.3	70	178.3	4.8	0.98	0.21	0.20
		5	228.1	97	182.5	5.3	0.80	0.19	0.15
II	6/5	2	272.8	54	147.9	2.5	–	0.40	0.22
		3	360.4	76	169.9	2.6	0.47	0.38	0.17
		4	396.9	95	185.0	3.0	0.48	0.33	0.16
		5	441.7	100	176.0	2.8	0.40	0.35	0.14
	T-1	2	129.1	32	128.8	3.1	–	0.32	0.32
		3	314.3	78.5	192.6	3.1	0.61	0.32	0.20
		4	342.4	92.5	220.2	3.4	0.64	0.30	0.19
		5	372.9	99	230.9	3.3	0.62	0.30	0.19
III	6/5	2	201.0	55.6	145.0	2.6	–	0.29	0.21
		3	315.0	77.6	182.6	2.3	0.58	0.32	0.19
		4	360.0	88.1	184.3	2.9	0.51	0.33	0.16
		5	401.5	95.3	176.0	2.9	0.49	0.34	0.15
	T-1	2	85.8	42.8	128.8	6.3	–	0.16	0.24
		3	262.0	76.8	166.4	3.6	0.64	0.27	0.17
		4	302.5	85.6	194.9	3.5	0.65	0.29	0.18
		5	363.0	94.5	218.1	3.2	0.60	0.31	0.18

*$\frac{\text{Biomass synthesis, mg}}{\text{Sucrose consumption, mg.}}$ †$\frac{\text{Acid formation, mg}}{\text{Sucrose consumption, mg.}}$

Table 2. Over-all Chemical Composition of Mycelium in Growing Cultures of Parent and Experimental Strains (in % of mycelium dry weight)

Age (day)	Total nitrogen		Total phosphorus		Ash	
	parent strain 6/5	variant T-1	parent strain 6/5	variant T-1	parent strain 6/5	variant T-1
2	7.27	8.82	0.86	1.21	2.49	2.74
3	5.85	7.17	0.61	0.82	1.85	3.07
4	5.51	5.46	0.47	0.61	1.35	1.76
5	4.39	4.99	0.32	0.48	0.55	1.09
7	3.91	3.86	0.22	0.31	0.29	0.58
9	3.80	3.79	0.13	0.25	0.20	0.40

Table 3. Amount of Phosphates in Various Fractions of Aspergillus niger 6/5 and Variant T-1 Mycelium (data are in μ/mg pellicle dry wt. on the 2nd day of growth on unchanged Zhuravskii medium)

Phosphorus	Parent strain 6/5	Variant T-1	Variant/ parent ratio
Acid-soluble			
Orthophosphate	0.25	0.31	1.24
Labile phosphate	0.66	0.75	1.13
Stable phosphate	0.75	0.83	1.10
Acid-insoluble			
Phospholipids	0.18	0.24	1.33
Nucleic Acids	2.00	2.77	1.38
Nonnucleoid phosphate (polyphosphate)	1.08	1.15	1.06
Precipitate after $HClO_4$ extraction (phosphoproteins)	0.78	1.13	1.45

ent strain by slower growth, increased acid formation, and somewhat greater sucrose consumption per unit weight of mycelium.

2. Comparative study of the over-all chemical composition of mycelium showed that the experimental strain differs from the parent strain in its increased phosphorus, nitrogen, and ash content, particularly in early growth stages.

3. Mycelium of the experimental strain contains a larger amount of phosphorus compounds in the various fractions than the parent strain.

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UTILIZATION OF ACETIC ACID AS SOLE CARBON SOURCE BY THE PHOTOSYNTHESIZING BACTERIUM, CHROMATIUM VINOSUM

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Utilization of various organic compounds by the purple bacteria has long been recognized to occur only in the case of members of Athiorhodaceae.

The Chromaceae, as typical autotrophic bacteria, have been considered incapable of living on organic products (Kondrat'eva, 1954, 1956; Elsden, 1954). Only after studies appeared from Shaposhnikov's laboratory (Nefelova, 1955; Shaposhnikov, 1957), which established for the first time the utilization by a Chromatium vinosum culture of certain organic acids when carbon dioxide, other organic compounds, or H_2S was present in the medium, did this conception begin to be modified. Until recently investigators have preferred to use the group Athiorhodaceae in studies on utilization of organic acids by purple bacteria (Clayton, Ellingston and Shaw, 1957; Clayton, Dettmer and Robertson, 1957; Clayton, 1957; Scardovi, 1955), since it is experimentally more convenient. Being rather unfastidious toward cultural conditions, it rapidly and invariably accumulates biomass, whereas the Chromaceae are very capricious. Yet a study of acetic acid utilization and the conditions of its utilization by the photosynthesizing sulfur bacteria, Chromaceae, is of great theoretical interest in determining the pathways of carbonic acid conversion in the process of carbon-skeleton formation in bacterial photosynthesis.

According to existing views (Shaposhnikov, 1957), the first steps in carbonic acid conversion during bacterial photosynthesis lead to formation of acetic acid (or its oxidation products). Then, acetic acid conversion by the photosynthesizing bacteria may proceed either along the pathway of oxidation to glyoxalic acid, glycolic acid, or glyoxal, or along the pathway of conversion to dicarboxylic acids—succinic, fumaric, oxalic. Foster (1954), in discussing the possibility of utilization of organic compounds by Thiorhodaceae, expresses the opinion that direct assimilation of organic compounds by the bacteria does not occur, and that organic compounds are first oxidized to CO_2 which the bacteria assimilate in the usual way.

Nefelova (1955) found, however, that acetic acid is utilized by Chromatium vinosum when another acid, CO_2 or H_2S, is simultaneously present in the medium. When acetic acid and H_2S are present together in the medium the acetic acid carbon consumed, with small losses, is converted to carbon of bacterial body substance. It was also noted that less H_2S is consumed than in growth on ordinary van Niel medium. The author suggests that acetic acid assimilation without previous oxidation to CO_2 can be assumed in this case.

Shaposhnikov (1957) believes that in the presence of H_2S the conversion of acetic acid proceeds by way of reduction to acetic aldehyde through H_2S oxidation to sulfuric acid. The acetic acid can also be utilized in other ways. The presence of condensation processes must first of all be assumed; in this case two acetic acid molecules may condense to form dicarboxylic acids—succinic, fumaric, etc. It is not unlikely that processes of simultaneous oxidation and reduction occur with formation of glyoxalic and glycolic acids as a result of oxidation of the acetic aldehyde methyl group by reduction of the COOH group.

It was shown in a previous report (Osnitskaya, 1958) that a Chromatium vinosum culture isolated from Lake Belo could multiply and accumulate biomass with organic acids as the sole source of carbon; this was established by determining the cell harvest of Chromatium vinosum grown on inorganic medium containing propionic, acetic, and certain other acids.

Since this may encounter the objection that, on the basis of increased number of cells alone, growth of the microorganisms on the substance consumed cannot be inferred, inasmuch as only division accompanied by decreased cell size may have occurred, it was necessary to confirm the observation by appropriate quantitative experiments.

Inasmuch as acetic acid conversion by photosynthesizing bacteria is of particular interest in connection with the problem of photosynthesis, experiments were first carried out on acetic acid utilization by Chromatium vinosum under different cultural conditions.

METHODS

Inorganic van Niel medium without $NaHCO_3$ was used. The medium was prepared, sterilized, and seeded with a culture suspension, with observation of precautionary measures to avoid the presence of traces of CO_2 from the air or water. Different variants of the experiment were set up: with acetic acid alone; with acetic acid and sodium sulfide; with acetic acid and $NaHCO_3$; with acetic acid, sodium sulfide, and $NaHCO_3$. Cultures were grown anaerobically in an illuminated thermostat at 30 deg; during the process of growth the appearance was observed of the bright red pigment characteristic of this culture. In van Niel medium, with $NaHCO_3$, acetic acid was added in the amount 0.2% as the sodium salt. pH of the medium was brought to 7.38-7.4 prior to seeding. Concentration of the culture suspension added did not exceed 200,000 to 300,000 per milliliter of medium. Analyses were made at two intervals in most of the experiments in order to observe the dynamics of the process. In experimental and control vessels taken for analysis, acetic acid was determined by distillation of volatile acids and fractional distillation according to Du Claux; cell yield and biomass, pH, CO_2 in the medium by the method of Friedmann and Kendall; cell carbon by the same method; hydrogen sulfide iodometrically; and sulfuric acid by the benzidine method.

Experimental results are given in the following tables and figures.

Table 1. Acetic Acid Consumption by Chromatium vinosum under Different Cultural Conditions

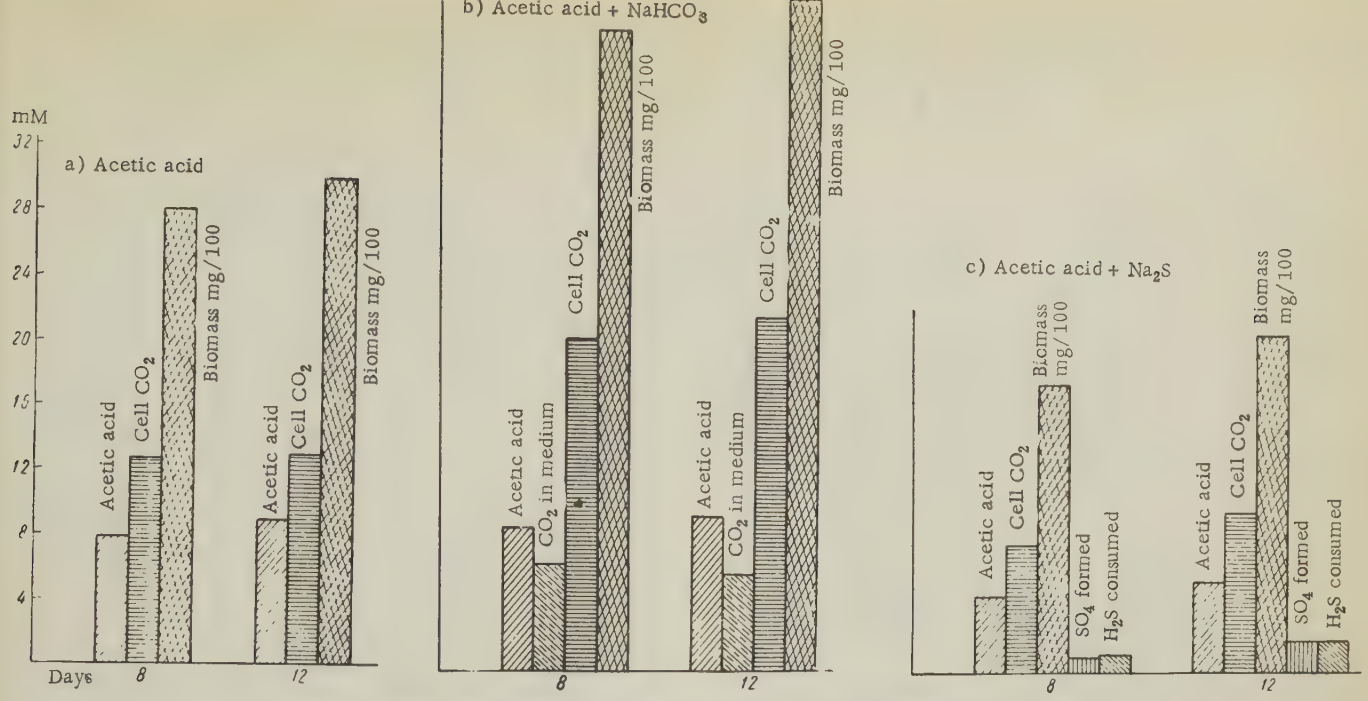
Inorganic van Niel medium without Na_2S or $NaHCO_3$ with addition of:	Growth (days)	pH	Biomass mg/100	Consumed, mm		Cell carbon expressed in mM CO_2	H_2S consumed, mM	H_2SO_4 formed, mM	mM H_2S/ mg cell C
				Acetic acid	CO_2 from medium				
Acetic acid	8	8.9	28	7.8	–	12.7	–	–	–
	12	9.0	30	9.0	–	13.0	–	–	–
Acetic acid + Na_2S	8	8.4	18	4.8	–	8.0	1.2	1.1	0.11
	12	8.8	21	5.5	–	10.0	2.0	1.99	0.16
Acetic acid + $NaHCO_3$	8	8.0	40	9.1	6.8	20.9	–	–	–
	12	9.1	42	9.8	6.3	22.3	–	–	–

Table 1 shows that the culture grew well in all the experiment variants: on acetic acid alone, on acetic acid with $NaHCO_3$, somewhat less well on acetic acid plus Na_2S with respect to both biomass production and acetic acid consumption. Alkalinization of medium to pH 8-9 is observed during growth in all variants. No sharp difference in rate of acetic acid consumption between experiments with acetic acid alone and those with acetic acid plus $NaHCO_3$ was observed. The amount of acetic acid consumed in these variants was 8-9 mM and was about 5 in the experiment with acetic acid plus Na_2S. In amount of biomass produced, culture growth in the variant with acetic acid plus $NaHCO_3$ (42 mg/100 ml) exceeded that on acetic acid alone (30 mg/100 ml). Together with the acetic acid consumption here, the consumption of up to 6.8 mM of CO_2 from $NaHCO_3$ occurred. Determination of cell carbon showed (Fig.) that the bulk of the carbon consumed from the medium is converted to cell carbon. Thus, on medium containing acetic acid, 7.8 mM of acetic acid was consumed in 8 days; when calculated as CO_2, 12.7 mM CO_2 was found in the cells. On medium containing acetic acid plus Na_2S, 4.8 mM acetic acid was consumed and 8 mM CO_2 was found in the cells. A larger discrepancy is observed on the medium containing acetic acid plus $NaHCO_3$, where 9.1 mM acetic acid and 6.8 mM CO_2 were consumed and the cells contained, when converted to CO_2, 20.9 mM. Thus a certain amount of carbon is to be found in the medium in the form of intermediate or secondary metabolic products.

It may be assumed that in this case part of the acetic acid is oxidized to glycolic or glyoxalic acid or to the dicarboxylic acids, fumaric and succinic. Another part is correspondingly reduced. Therefore, it happens that acetic acid consumption exceeds formation of bacterial body carbon. Prolonging experiments from 8 to 12 days exerted no particular effect on the process: The main production of biomass and consumption of carbon from the medium occurred during the first 8 days; considerably less consumption of carbon-containing compounds and biomass production occurred during the last 4 days. Thus, on medium containing acetic acid, 7.8 mM acetic acid was consumed in 8 days and biomass was 28 mg. In 12 days the biomass was 30 mg and 9.0 mM acetic acid was consumed: The same phenomenon was observed in the variant with acetic acid plus $NaHCO_3$, were consumed and biomass was 40 mg, and in 12 days 9.8 mM acetic acid and 6.3 mM CO_2 were consumed and biomass was 42 mg. In the variant with acetic acid plus Na_2S, low consumption of H_2S (1-2 mM) occurred, and an equivalent formation of SO_4. In the experiments with acetic acid and with acetic acid plus Na_2S, no appearance of determinable amounts of CO_2 was noted in the medium in the course of the experiments.

On the basis of the data examined it can be said that acetic acid is utilized by our Chromatium vinosum culture when it is the sole carbon source and regardless of whether H_2S or $NaHCO_3$ is present in the medium, the bulk of the carbon consumed being found in the bacterial cell substance. At the same time a certain amount of the carbon obviously remains in the medium in the form of intermediate or secondary products. The presence of an oxygen acceptor, H_2S, in the medium is not necessary; the culture utilizes it poorly and there is no comparison with the H_2S consumption on ordinary van Niel medium containing $NaHCO_3$, where all the H_2S is consumed in the first 2-3 days. On "acetic acid + Na_2S" medium about 0.08-0.11 mM H_2S was consumed per one assimilated carbon and on "acetic acid + Na_2S + $NaHCO_3$" medium about 0.22-0.25 mM, whereas this value on van Niel medium was usually 0.5-0.7 mM in the first 2-3 days. This difference in H_2S consumption is determined by the greater state of reduction of the acetic acid carbon compared to CO_2 carbon. Somewhat longer experiments did not affect the general course of the process or the conclusions that can be drawn from the data obtained. This series of experiments gave quite a clear picture of the fact that, according to acetic acid consumption and biomass production, the basic culture growth is completed in 8-10 days. We also conducted longer experiments (up to 30 days) in order to determine what changes would be observed in this case in the process of culture growth.

The data from these experiments is given in Table 2. It follows from the table that the most active biomass production and consumption of carbon sources, with conversion of the bulk of the carbon consumed to carbon in bacterial bodies, occurs in the first 10 days and then, despite the presence of considerable amounts of carbon source in the medium, as well as H_2S in experiments where Na_2S was added, the culture grows considerably more slowly as indicated by the insignificant, compared to the first 10 days, biomass production and



Acetic acid utilization, biomass production, and cell carbon at different growth intervals.

Table 2. Acetic Acid Consumption by Chromatium vinosum with Varying Duration of Experiment

Inorganic van Niel medium without Na_2S or $NaHCO_3$ with addition of:	Growth (days)	pH	Cell harvest, million/ml	mM consumed		Cell carbon expressed in mM CO_2	mM H_2S consumed	mM H_2SO_4 formed	mM H_2S/mg cell C
				Acetic acid	CO_2 from medium				
Acetic acid	10	8.2	72.6	7.0	–	14.8	–	–	–
	30	8.9	82.6	12.7	–	13.2	–	–	–
Acetic acid + Na_2S	10	8.7	129.0	9.8	–	20.5	1.5	1.2	0.06
	30	7.7	120	13.0		18.1	2.5	2.5	0.10
Acetic acid + $NaHCO_3$ + + Na_2S	10	7.4	180.0	6.9	1.3	12.7	3.4	0.5	0.22
	30	7.3	172.9	7.0	4.7	15.2	4.1	1.0	0.22

acetic acid consumption. After 30 days there was also observed an incomplete correlation between amount of carbon assimilated from the medium and carbon in bacterial bodies. Thus, after 30-day culture growth on acetic acid, 12.7 mM of acetic acid was consumed and carbon in bacterial bodies, calculated as CO_2, was only 13.2 mM. On acetic acid plus Na_2S, the cell carbon content after 30 days was 18.7 mM CO_2 and acetic acid consumed reached 13 mM. In other words, part of the acetic acid carbon remained in the medium in the form of intermediary or secondary products.

As for H_2S, even in these long experiments it was very poorly utilized and considerable amounts were found in the medium at the end of the experiment.

The question of the effect of amount of seed material added to medium was also incidentally determined. It was found that culture growth takes place only when an adequate number of cells are present. A normal growth picture was observed in our culture and under our experimental conditions when the bacterial concentration in the medium after addition of the seed material was 200,000-400,000 cells per ml. Decreasing the bacterial concentration to 100,000 per ml produced very great delay and sometimes complete absence of growth even in the experimental variants in which acetic acid was added together with $NaHCO_3$ and Na_2S or on classical van Niel medium. In cases where growth did occur, the process of utilizing carbon and H_2S in the medium proceeded in the same way that we have already indicated. As an illustration, data are given in Table 3 from experiments with decreased concentration of added seed material (to 100,000 per ml of medium).

Visible growth started in this experiment only after a lapse of 15 days. Samples were taken after 20 and 22 days.

As seen from Table 3, considerable amounts of acetic acid were consumed in this case also.

It should be noted that the experimental seed material suspension was prepared from a regular culture transfer on van Niel medium containing $NaHCO_3$. Thus the culture was by no means previously "adapted" to acetic acid consumption by repeated transfers on medium containing acetic acid. On the contrary, the culture always grew excellently on van Niel medium containing $NaHCO_3$ as a typical photosynthesizing autotroph.

Table 3. Chromatium vinosum Growth and Acetic Acid Consumption when Medium is Seeded with Smaller than Usual Amounts of Culture

Inorganic van Niel medium without Na_2S or $NaHCO_3$ with addition of:	Growth (days)	pH	Cell harvest, million/ml	mM consumed		Cell carbon expressed in mM CO_2	mM H_2S consumed	mM H_2SO_4 formed	mM H_2S/mg cell C
				Acetic acid	CO_2 from medium				
Acetic acid + Na_2S	20	7.8	53.5	6.3	–	12.3	1.2	1.13	0.08
	22	7.8	55.0	6.1	–	12.7	1.33	0.96	0.08
Acetic acid + Na_2S + $NaHCO_3$	20	7.7	84.4	6.6	12.5	14.0	4.20	3.2	0.25
	22	8.0	91	6.1	7.2	14.3	4.21	3.2	0.25

SUMMARY

1. Quantitative experiments showed that the photosynthesizing bacterium, Chromatium vinosum, can utilize acetic acid as sole carbon source during development under anaerobic conditions in the presence of light.

2. In the initial growth period (8-12 days) the acetic acid carbon is obviously used chiefly to build bacterial bodies, as indicated by correspondence between the amount of acetic acid carbon consumed and cell carbon.

3. During further development, owing to setting in of autolysis, biomass production and acetic acid conversion are slowed and correspondence between cell carbon and acetic acid carbon consumed is not observed.

4. When $NaHCO_3$ is present in the medium together with acetic acid, a decrease in amount of CO_2 in the medium was also noted.

5. H_2S consumption in experiments with acetic acid or with acetic acid + $NaHCO_3$ was insignificant, amounting to 0.08-0.11 mM per one assimilated carbon for acetic acid + Na_2S, and 0.22-0.25 mM for acetic acid + $NaHCO_3$ + Na_2S; H_2S remained in the medium even in lengthy experiments which was never the case when the culture was grown on van Niel medium containing $NaHCO_3$ where 0.5-0.7 mM H_2S per one assimilated carbon is consumed in the first few days of growth.

6. Decreasing the amount of inoculum to 100,000 per ml or less greatly retards growth and sometimes renders it altogether impossible.

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THE TRICARBOXYLIC ACID CYCLE IN MOLDS

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The lack of experimental data giving a picture of the further oxidation of acetic acid has tended to cause investigators to bypass this difficulty, assuming that acetic and pyruvic acids do not undergo direct oxidation but are oxidized only after combining with oxaloacetic acid.

The scheme of carbohydrate oxidation, proposed by Krebs and Johnson in 1937, has been widely disseminated in recent times. It includes the hypothesis of Szent-Gyorgyi. Krebs (1943) proposed the hypothesis termed the citric acid cycle or tricarboxylic acid cycle.

According to this hypothesis, pyruvic acid formed in the anaerobic phase of carbohydrate breakdown does not undergo direct oxidation during respiration, but is oxidized only after combining with a molecule of oxaloacetic acid. The citric acid formed is oxidized through α-ketoglutaric, succinic, and fumaric acids to oxaloacetic acid. Thus, in the course of a single cycle, one molecule of pyruvic acid is oxidized and one molecule of oxaloacetic acid is simultaneously regenerated.*

In the opinion of W. James (1956), if the respiration of an organism or tissue proceeds to any considerable extent through the tricarboxylic acid cycle, then the following conditions should obtain: 1) Presence of intermediary products of the cycle; 2) presence of the appropriate enzymes; 3) relatively large increase in oxygen absorption without change in respiration coefficient upon addition of small amounts of intermediary products; 4) cessation of oxygen absorption after a short induction period upon addition of approximately M/40 malonate; 5) succinic acid formation through oxidation of a pyruvic and fumaric acid mixture, if succinic acid formation through fumaric acid reduction has been inhibited by malonate; 6) succinic acid formation under analogous conditions from malic and oxaloacetic acids.

We would on our part add still another condition that should also obtain in the presence of a full tricarboxylic acid cycle in microorganisms, namely: oxidation of a substrate, for example glucose, through fumarate or oxaloacetate reduction should occur, as was established for Bact. coli by Krebs (1937).

James (1956) considers that none of these characteristics alone can demonstrate the presence of the tricarboxylic acid cycle, since each may be produced by other causes.

There are many studies in the literature (Martins and Lynen, 1950; Clark and Wallace, 1958) where it is asserted on the basis of one or two characteristics that the metabolism of the material studied proceeds via the tricarboxylic acid cycle.

*Since fungi were grown on glucose, the fundamentals of the tricarboxylic cycle are reviewed in this paper.

Remakrishnan (1954) found enzymes of the tricarboxylic acid cycle in cell-free extracts of Aspergillus niger.

In L'vov's opinion (1950), the theory of the Krebs cycle is still a controversial one.

Krebs, Gurin, and Eggleston (1952), on the basis of their own experiments with yeast cells and studies of other investigators with bacteria, reached the conclusion that in yeast cells, as well as in many other organisms, the reactions of the tricarboxylic cycle have no relation to respiration, but serve to provide compounds used in synthetic processes.

Wiame and Bourgeois (1953) confirmed Krebs' conclusions and showed that none of the compounds of the tricarboxylic acid cycle participates in the respiration of Bac. subtilis, but that these substances increase the rate and maximum growth of this bacterium. The authors concluded on the basis of these observations that the biological significance of the tricarboxylic acid cycle is associated with a formation of glutamic and aspartic acids. In Mikhlin's opinion (1956), however, the biological significance of the tricarboxylic acid cycle is not sufficiently clear despite the great number of experimental data. James (1956) also considers there to be very little evidence in favor of plant acids forming a catalytic oxidation cycle and, on the contrary, data exist that contradict the suggestion of the existence of the cycle in certain plant species.

There are few data on the presence in molds of the tricarboxylic acid cycle as the main pathway of carbohydrate oxidation.

Foster (1949) points out that the tricarboxylic acid cycle has not yet been studied in fungi, although the oxidation of citric acid itself apparently proceeds through this cycle.

Krebs (1943) considers the mechanism of citric acid decomposition by aspergillus to be little known, since molds, in distinction to bacteria and animal tissues, can form oxalic acid during citric acid oxidation.

The absence of the Krebs cycle in A. niger is also indicated by experiments of Fencl (1958), where he showed that aconitase inhibition by fluoroacetic acid causes no increase in citric acid yield.

Novak (1958) found malonic acid consumption in the mold Botryotinia fuckeliana and showed that malonic acid in 0.05, 0.10, and 0.15% concentration accelerates growth of B. fuckeliana twofold on Czapek-Dox medium containing sucrose. These facts, in Novak's opinion, speak against the existence of the Krebs cycle in B. fuckeliana.

It is possible to interrupt the tricarboxylic acid cycle by inhibiting any reaction of the cycle and thus halt respiration and growth of the organism under study. Several substances are now known to inhibit reactions in the cycle. One of these inhibitors is malonic acid,

a basis for claiming the presence of a complete tricarboxylic acid cycle, since it has not been demonstrated that the succinic dehydrogenase does not exist outside the cycle.

We already pointed out, one of the indications that the respiration of a given organism proceeds via the tricarboxylic acid cycle is the cessation of oxygen consumption after a short induction period upon addition of approximately 0.025 M malonic acid, owing to inhibition of oxaloacetic acid formation during succinic acid dehydrogenation by succinic dehydrogenase. Oxaloacetic acid should be continuously formed in the metabolism of growing cells, since, according to the statement of Krebs, Gurin, and Eggleston (1952) and Wiame and Bourgeois (1953), growing cells utilize compounds of the cycle for synthetic processes.

In order to determine whether the reactions of the tricarboxylic acid cycle are the main pathway of carbohydrate oxidation in fungi, we cultivated R. nigricans and A. niger on nutrient medium containing 0.02-0.06 M/liter malonic acid. The fungi were grown on the media whose composition was indicated under "Methods" and whose reaction was weakly acid, i.e., under conditions providing for entrance of malonic acid into the fungus cells. Since malonic acid is partially decomposed by heating it was dissolved in the experiments, in sterile distilled water, neutralized with sodium hydroxide to the pH of the nutrient medium, and added with sterile precautions to the media prior to seeding. To media not containing malonic acid, glucose, corresponding to the highest malonic acid concentration, was also added. Phosphate buffer of appropriate acidity was added to the medium to stabilize the reactions, its final concentration being 0.05 M. Duration of the experiment was 24 hours.

Table 3. Effect of Malonic Acid on Growth of Rhizopus nigricans and Aspergillus niger

Malonic acid M/liter	R. nigricans grown on 2% glucose medium pH 5.3 at 25 deg			A. niger grown on 1% glucose medium pH 4.6 at 27 deg		
	pH after expt.	fungus wt.		pH after expt.	fungus wt.	
		mg	%		mg	%
0	2.4	102.5	100.0	2.7	58.1	100.0
0.02	3.0	106.4	103.9	3.2	57.8	99.5
0.04	3.8	102.7	100.2	3.4	73.1	125.8
0.06	4.2	109.1	106.4	3.7	79.2	138.0

The experimental results given in Table 3 show that 0.02 M malonic acid, which completely inhibits succinic dehydrogenase in experiments by the method of Thunberg, does not inhibit growth of R. nigricans and A. niger. Even a malonic acid concentration as high as 0.06 M does not affect growth of R. nigricans, and A. niger grows even faster in the presence of 0.04 and 0.06 M malonic acid than in the control, probably owing to relative pH stabilization. All this leads to the conclusion that succinic dehydrogenase does not participate in the metabolism proceeding during the growth of these fungi on glucose and, consequently, the reactions postulated in the tricarboxylic acid cycle do not occur either in respiration or in supplying these fungi with the intermediary compounds utilized during their growth on glucose. Consequently our results do not confirm the conclusions of Krebs and of Wiame and Bourgeois.

Table 4. Effect of Malonic Acid on Growth of Aspergillus niger
(Duration of experiment 42 hours at 25 deg)

pH before expt.	Nutrient medium	pH after expt.	Fungus wt., mg
4.2	0.04 M/liter glucose	2.8	55.4
3.9	0.04 M/liter glucose and 0.04 M/liter malonic acid	3.2	54.4
3.7	0.04 M/liter malonic acid	3.7	0.0
4.6	0.04 M/liter malonic acid	4.6	0.0
5.7	0.04 M/liter malonic acid	5.7	0.0

As was already pointed out, Novak found malonic acid consumption in the mold, B. fuckeliana.

In order to determine whether the mold, A. niger, utilizes malonic acid, the fungus was grown on medium containing 0.04 M/liter malonic acid and a small glucose supplement (0.04 M/liter), for a relatively long time (42-96 hours), until all the glucose was consumed. Supplementary glucose was not added to control flasks without malonic acid. Malonic acid remaining in the medium was determined by oxidation with potassium permanganate.

It is evident from the experiment shown in Table 4 that A. niger does not grow on malonic acid alone and that the fungus yield is the same on media with glucose alone and with glucose plus malonic acid. Consequently, although all the sugar in the medium was utilized by the fungus, growth did not occur through malonic acid consumption. Furthermore, the determinations of malonic acid by potassium permanganate oxidation at the end of the experiment showed that it is not utilized by the fungus.

3. Succinic Dehydrogenase Participation in Fumaric Acid Formation in Rhizopus Nigricans on Glucose

In order to clarify the question of succinic dehydrogenase participation in fumaric acid formation, we conducted experiments in which previously grown R. nigricans pellicles were placed in glucose solution containing malonic acid.

R. nigricans pellicles were grown 4 days, as indicated under "Methods", then the nutrient solution was poured off from under the pellicle and the pellicle was washed three times with 1% sodium sulfate solution. The fungus was then starved for 3 hours in 1% sodium sulfate solution containing malonate in the amounts employed in the experiment. After 3 hours of starvation the mixture was poured off and under the pellicle was added 45 ml of 10% glucose solution in phosphate buffer (0.1 M pH 6.5) containing malonic acid. Since the fungus ferments glucose to organic acids quite rapidly, the medium under the pellicle, despite the presence of

which has been found to inhibit specifically the dehydrogenation of succinic acid by succinic dehydrogenase. We have used this reaction of succinic dehydrogenase inhibition by malonic acid in our study to settle the question of the presence of the tricarboxylic cycle in A. niger and Rhizopus nigricans.

We judged the tricarboxylic cycle to be present in fungi, firstly, from the presence of succinic dehydrogenase determined according to Thunberg and from its inhibition by malonate, secondly, from succinic dehydrogenase participation in the metabolism of fungi growing on glucose-containing media, and finally, from its participation in fumaric acid formation in R. nigricans.

METHODS

In Experiments 1 and 3 R. nigricans was grown for 4 days at 30-31 deg in 100-ml flasks on nutrient medium containing (in %): glucose 5; $(NH_4)_2SO_4$ 0.3; KH_2PO_4 0.003; $NaH_2PO_4 \cdot H_2O$ 0.063; $MgSO_4 \cdot 7H_2O$ 0.03; $FeCL_3 \cdot 6H_2O$ 0.003; $ZnSO_4 \cdot 7H_2O$ 0.005; and calcium carbonate 1. The latter was sterilized separately and added during seeding.

In Experiment 2 R. nigricans, and A. niger in Experiments 1 and 2, were grown in submerged culture on a shaker for 24-48 hours on medium containing (in %): glucose 1–2; $NH_4H_2PO_4$ 0.052; K_2SO_4 0.0424; $MgSO_4 \cdot 7H_2O$ 0.03; $FeCl_3 \cdot 6H_2O$ 0.003; $ZnSO_4 \cdot 7H_2O$ 0.005 and $MnSO_4 \cdot 7H_2O$ 0.003.

All experiments were conducted in 2-3 parallel flasks. Mycelium at the end of the experiment was filtered off on a glass filter, washed, and dried at 80 deg to constant weight. Glucose was determined according to Bertrand. Alcohol was distilled off and oxidized with potassium dichromate by a modified method of Kuriloff (1897). Organic acids were extracted with ether. Fumaric acid was determined, after destruction of malonic acid, as the mercury salt by the method of Olander (1929). After malonic acid destruction and fumaric acid oxidation with permanganate, succinic acid was extracted with ether and titrated with alkali.

1. Detection of Succinic Dehydrogenase in Rhizopus Nigricans and Aspergillus Niger According to Thunberg and Its Inhibition by Malonate.

To establish the presence of succinic dehydrogenase, R. nigricans was grown as indicated above. The pellicle was washed with water, dried between sheets of filter paper, and lightly ground. The ground pellicle was suspended in phosphate buffer (pH 7.6), 20 mg crude weight of pellicle per ml buffer. A. niger was grown on the above-mentioned medium containing 2% glucose at 30--31 deg for 24 hours on a shaker, the mycelium was filtered off, twice washed with distilled water, dried between sheets of filter paper, and suspended in phosphate buffer (pH 7.4), 100 mg crude weight of fungus per ml buffer. Succinic and malonic acids were used in the form of potassium salts. Methylene blue reduction was carried out at 37 deg.

The experimental results presented in Tables 1 and 2 show that R. nigricans and A. niger reduce methylene blue in media without substrate, i.e., as a result of

Table 1. Determination of Succinic Dehydrogenase in Rhizopus nigricans by Methylene Blue Reduction and Effect of Malonic Acid on the Succinic Dehydrogenase [Methylene blue 1 ml (1 : 20,000); 20 mg crude weight of mycelium]

Substrate	Malonic acid, M/liter	Methylene blue reduction time in min.
Without substrate	0	18
0.1-M succinic acid	0	5
0.1-M succinic acid	0.005	10
0.1-M succinic acid	0.02	17
0.1-M succinic acid	0.06	30

Table 2. Determination of Succinic Dehydrogenase in Aspergillus niger by Methylene Blue Reduction and Effect of Malonic Acid on the Succinic Dehydrogenase [Methylene blue 1 ml (1 : 10,000); 100 mg crude weight of mycelium]

Substrate	Malonic acid, M/liter	Methylene blue reduction time in min.
Without substrate	0	115
Without substrate	0.02	125
0.02-M succinic acid	0	53
0.02-M succinic acid	0.02	125

compounds in the mycelium. It is further evident that R. nigricans and A. niger reduce methylene blue much faster in media containing succinic acid. The increase of 2--3.6 times in methylene blue reduction produced by succinic acid indicates the presence of succinic dehydrogenase in the fungi investigated. It is also seen from the tables that 0.005 molar malonic acid causes a twofold decrease in methylene blue reduction rate by R. nigricans, and 0.02 M malonic acid completely inhibits the methylene blue reduction by R. nigricans and A. niger due to dehydrogenation of the added succinic acid. On increasing malonic acid in the medium to 0.06 M, the rate of methylene blue reduction is even lower than that in media without the substrate. Therefore 0.06-M malonic acid also inhibits, besides succinic dehydrogenase, other dehydrogenases that participate in methylene blue reduction by substances in the mycelium.

Thus the results obtained permit the conclusion that succinic dehydrogenase is present in R. nigricans and A. niger and that it is completely inhibited by 0.02-M malonic acid. This conclusion is in full accord with data existing in the literature.

2. Succinic Dehydrogenase Participating in Fungus Metabolism.

In the opinion of James (1956), the presence of succinic dehydrogenase in R. nigricans and A. niger is not

buffer, is very rapidly acidified to pH 4.5-5.0, and therefore, the reaction under the pellicles was regularly adjusted to pH 6.5 with sodium hydroxide during the experiment.

Table 5. Effect of Malonic Acid on Growth of Rhizopus nigricans and on Its Formation of Alcohol, Fumaric Acid, and Succinic Acid

Malonic acid conc., M/liter	Glucose consumed, mg	Fungus wt., mg	Alcohol, mg	Fumaric acid, mg	Succinic acid, mg
–	–	Before expt. 178	–	–	–
0	2856	262	223	1085	49
0.005	2904	286	229	1070	181
0.02	2937	264	233	1296	151
0.06	3055	318	223	1310	212

It is seen from Table 5 that when R. nigricans is transferred from nutrient solution to glucose solution containing malonic acid, it continues to grow, the fungus yield even being 21.3% greater on the medium containing 0.06 M malonic acid. It is further seen that malonic acid does not affect glucose consumption or alcohol formation.

As for the effect of malonic acid on the process of fumaric and succinic acid formation, as seen from Table 5, 0.02-0.06 M malonic acid increases fumaric acid production by 19-20%. Succinic acid is formed in R. nigricans cultures in small amounts, about 50 mg; its amount in control cultures is 22 times less than that of fumaric acid. Succinic acid formation in cultures containing malonic acid increases 4.3 times, reaching 212 mg, but even in this case the amount is one sixth that of fumaric acid. Consequently, production of fumaric and succinic acids simultaneously increases on media containing malonic acid. This fact indicates that these processes are independent of one another. According to the tricarboxylic acid cycle, however, only succinic acid can accumulate in cultures on glucose containing malonic acid, since its dehydrogenation to fumaric acid is inhibited by the malonic acid. Consequently, succinic dehydrogenase does not participate in this process and fumaric acid is not formed via the Krebs cycle, i.e., by succinic acid dehydrogenation.

We consider it necessary to point out that in one of our studies (Barinova, 1941), we found fumaric acid and alcohol in equimolecular amounts. This prompted the suggestion that the formation of these compounds results from breakdown of glucose to C_2 and C_4 compounds.

SUMMARY

1. Aspergillus niger and Rhizopus nigricans contain succinic dehydrogenase.

2. Methylene blue reduction by succinic dehydrogenase of A. niger and R. nigricans is completely inhibited by 0.02 M malonic acid.

3. Malonic acid in 0.02-0.06 M concentration does not inhibit growth of R. nigricans and A. niger.

4. A. niger does not utilize malonic acid; the latter is not consumed by the mold.

5. Succinic dehydrogenase does not participate in the metabolism of R. nigricans and A. niger growing on glucose.

6. The tricarboxylic acid cycle plays no role in respiration and metabolism of R. nigricans and A. niger growing on glucose.

7. Malonic acid in 0.005-0.06 M concentration in the medium does not inhibit fumaric acid formation and increases succinic acid formation in R. nigricans on glucose.

8. Succinic dehydrogenase does not participate in fumaric acid formation.

9. Fumaric acid is not formed via the tricarboxylic acid cycle, i.e., through succinic acid dehydrogenation, in R. nigricans on glucose.

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GROWING IN A PLAQUE ON VAN NIEL MEDIUM

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Despite the fact that spirilla are widely prevalent aquatic microorganisms, the physiology of many of them has been studied very slightly.

Thus a large number of saprophytic forms of spirilla have been described (Bergey, 1936; Krasil'nikov, 1949; Bergey, 1948); however, virtually nothing is known of the processes carried out by these organisms in the water. Those studied in greatest detail appear to be the spirilla belonging to the group of sulfate-reducing bacteria. Regarding this group there is a great deal of work; in particular, the ability of these microorganisms to shift from a heterotrophic to an autotrophic mode of life has been studied in detail (Butlin and Adams, 1947; Sorokin, 1953). The group of spirilla able to fix atmospheric nitrogen is of interest. These spirilla, first described by Beijerinck, appear to be widely prevalent in water (Rodina, 1956). The species studied by Rodina were found to be able to grow as ordinary heterotrophs.

A member of the purple nonsulfur bacteria, Rhodospirillum rubrum, isolated by van Niel, is an object of study in the field of comparative biochemistry and is of interest in connection with its combination of properties, such as the capacity for photosynthesis and the requirement for organic compounds to accomplish this.

There is quite a bit of information on the occurrence in water of colorless thiospirilla; however, there has been very little study of the properties of these organisms. The work of Lindforss (1912) is known, regarding chemotaxis in spirilla, which he had to collect several times from water since attempts to cultivate them in the laboratory were not successful. The most detailed studies are those of Egunov (1896a, 1895a, 1895b, 1896b, 1900, 1901), on the investigation of plaques formed by spirilla in cylinders containing bottom silt. Finally, there are the observations of Omelyanskii (1953) on a culture of Thiospirillum vinogradskii, likewise grown as a plaque in a cylinder containing silt and water.

We have studied the behavior of thiospirilla that form plaques when grown in test tubes containing agarized van Niel medium covered with water, and growing in association with accompanying microorganism forms (Vladimirova, 1958). The present paper gives the results of a study on the properties of this organism isolated in pure form.

METHODS

A pure culture of spirilla was obtained by a technique based on the method of growing and obtaining pure cultures of colorless filamentous sulfur bacteria, developed by Keil (1912). This method consists in selecting the proper ratios of gases in a mixture of CO_2, H_2S, and O_2.

The spirillum culture in our experiments was seeded in sterile 100 ml vessels containing liquid inorganic van Niel medium (25 ml), bound with sterile cotton gauze cloth instead of cotton stoppers for freer gas exchange. Vessels were put in a vacuum desiccator at the bottom of which were small beakers containing definite amounts of sodium bicarbonate and sodium sulfide. The necessary gas ratios were obtained by adding H_2SO_4 after evacuating air to the necessary pressure. Several variants were set up on van Niel medium with various pH values and various concentrations of H_2S, CO_2, and O_2 (varying oxygen content was achieved by stepwise evacuation of air from the desiccator). The atmosphere in desiccators was adjusted with nitrogen. Desiccators were kept in the dark at 19-23 deg for 1.5-2 weeks. No visible growth was found in a single case. When seedings were made in test tubes containing agarized van Niel medium covered with water, a spirillum culture was obtained in only one case. This occurred upon seeding from a was 20 mm Hg, H_2S concentration 25 mg/liter, no CO_2 was added separately, and pH of the medium was 6.5

Immediately after finding the growth of spirilla, a series of dilutions was made in test tubes. A check of these dilutions showed that we had a culture of spirilla entirely free of accompanying organisms. The check was made by seedings on various organic media (meat-peptone agar, meat-peptone gelatin, meat-peptone broth, potato agar, wort agar, liquid wort, liquid inorganic medium containing peptone, media for thiobacteria and sulfate-reducing bacteria). Neither growth nor the presence of individual cells of accompanying forms was found in a single case, but growth of spirilla was always observed.

A number of questions arose in connection with the fact that the spirilla proved to be capable of an ordinary heterotrophic mode of life. First of all it was necessary to determine whether we had a mixture of various species of spirilla in the original plaque and whether we had lost during isolation species capable of autotrophic life. Secondly, if autotrophic properties were retained, did we not have even now a mixture of autotrophic and heterotrophic species of spirilla, and was not the growth on organic media due to a purely heterotrophic spirillum species. Only when we were convinced of the retention of autotrophic properties by the spirilla grown on organic media, could we begin a study of the heterotrophic properties of this organism.

Experiments to Determine the Capacity of Spirilla for an Autotrophic Mode of Life.

In order to determine whether the isolated spirilla retained the capacity for autotrophism after purification, a series of experiments was set up on liquid inorganic van Niel medium in 50 ml flasks. Medium was added in 25 or 40 cm^3 amounts (as the experi-

ments showed, no essential difference was found here, except that H_2S was oxidized by atmospheric oxygen more actively in flasks with 25 ml of medium). Flasks were closed with rubber stoppers. Cultivation continued for 2 weeks in the dark in a thermostat at 25 deg. Prior to analysis, 2 ml of 1 N NaOH, CO_2-free, was added to the flask through the stopper with a syringe; flasks were opened, and the fluid analyzed for CO_2 (Van Slyke apparatus), H_2S (iodometrically), and H_2SO_4 (nephelometrically). Control flasks were set up in parallel, unseeded but with a cell suspension killed by boiling; these were kept under the same conditions for the same length of time. CO_2 consumption, H_2S oxidation, and H_2SO_4 formation were determined by the difference between control and experimental. Averages of duplicates or triplicates are given in all the following tables.

As preliminary experiments showed, the spirilla were capable of autotrophism only in the presence of 0.001-0.0001% yeast autolyzate and did not grow on purely inorganic medium. It is not a question, in this case, of heterotrophic nutrition by organic substances in the yeast autolyzate, since the amounts added were too slight and the aggregate of processes occurring indicates the capacity of these spirilla for an autotrophic mode of life. A requirement for vitamins should not be regarded as a denial of autotrophic properties (Woods and Lascelles, 1954).

In order to establish what part of the yeast autolyzate acts on the spirilla, an experiment was set up to compare the growth on purely inorganic medium, on medium containing whole yeast autolyzate, and on medium containing yeast autolyzate treated in different ways—adsorption on activated charcoal at acid pH and at alkaline pH (Ierusalimskii, 1949). Results are given in Table 1.

As seen from Table 1, approximately the same results are obtained in growth on whole yeast autolyzate and on that treated at pH 3. According to Ierusalimskii, yeast autolyzate treated under these conditions retains the following vitamins: thiamine, riboflavin, β-alanine, pyridoxine, i-inosite.

Of course the next step in the study was the attempt to replace yeast autolyzate with the indicated vitamins. Vitamins were sterilized as separate solutions at 0.5 atm for 15 minutes and added to sterile medium in optimal amounts. Results are given in Table 2.

As seen from the table, the changes in the medium during growth in the presence of vitamins were of the same order as on medium containing yeast autolyzate (Table 1). The growth on medium containing yeast autolyzate was, in the given experiment, generally much weaker.

Similar results in growth on this medium containing vitamins were obtained subsequently on double-distilled water with replacement of rubber stoppers by groundglass stoppers. In these experiments, the alkali was added through a special sidearm according to the method employed by Sorokin (1953, 1954).

Thus, the experiments performed indicate that the spirillum culture isolated, maintained in test tubes containing van Niel medium, is capable of an autotrophic mode of life, but for this, the spirilla require a certain amount of vitamins.

It should be noted that growth was very weak in all cases (both with autolyzate and with vitamins), so that to judge the growth from number of cells was not very reliable. But chemical analysis always gave entirely reproducible results. In cases where for some reason growth did not occur, we always obtained negative results in both the CO_2 determination (CO_2 usually increased in these cases) and the sulfate determination (the amount remained unchanged).

Do spirilla isolated from colonies on organic media and maintained on organic media possess these same properties? To answer this question, experiments of the same design were set up and the following variants of the spirillum culture were employed:

1. Spirilla obtained by repeated seeding on plates containing MPA and subsequent transfer from an individual colony to an MPA slant, and then to test tubes containing van Niel medium.

2. A culture obtained in the same way, but seeding on agarized wort 0.5 deg Balling.

3. A spirillum culture obtained by seeding liquid inorganic van Niel medium (without Na_2S or bicarbonate), with addition of 1% peptone and use of the dilution

Table 1. Growth of Spirilla on van Niel Medium in Relation to Nature of Yeast Autolyzate Added

van Niel medium	Expt. variant	CO_2 in mg/liter		H_2SO_4 in mg/liter	
		determined	consumed	determined	formed
Yeast autolyzate not added	Killed cells (control)	170	–	15.7	–
	Living cells (expt.)	170	0	18.0	2.3
+ whole yeast autolyzate	Killed cells (control)	191	–	17.3	–
	Living cells (expt.)	176	15	33.1	15.8
+ yeast autolyzate treated at pH 7.0–7.5	Killed cells (control)	187	–	14.5	–
	Living cells (expt.)	185	2	18.6	4.1
+ yeast autolyzate treated at pH 3	Killed cells (control)	196	–	14.8	–
	Living cells (expt.)	181	15	26.0	11.2

Yeast autolyzate was added in the amount 0.001% by volume in all cases.

Table 2. Growth of Spirilla on van Niel Medium Containing Added Vitamins

Expt. variant	CO_2 in mg/liter		H_2SO_4 in mg/liter	
	determined	consumed	determined	formed
Killed cells (control)	677	–	41.1	–
Living cells (expt.)	642	35	54.0	12.9

method on this medium and a large number of passages (2-3 months). This culture, too, was seeded in test tubes containing ordinary van Niel medium.

Experimental results on the study of the autotrophic properties of these cultures are given in Table 3. The cultures from MPA and peptone were studied simultaneously; experiments were set up on medium containing vitamins and seedings were made with the same number of cells. Later, the culture from wort was also studied on medium containing yeast autolyzate.

It is seen from the table that the cultures, maintained for a time on organic medium, retain the capacity for autotrophic existence on inorganic van Niel medium containing vitamins, since H_2S is oxidized on this medium with formation of sulfuric acid and CO_2 consumption occurs.

Since the first experiments on the ability of spirilla to oxidize H_2S to sulfate were carried out with a mixed culture in the presence of accompanying organisms, and in cylinders containing agarized van Niel medium under a water layer with the growth of a plaque (Vladimirova, 1958), it was of interest to check whether the spirilla would remain active under the same conditions after purification from associated organisms. This was particularly interesting since the type of growth of the pure culture in test tubes had changed sharply; growth became much slower and was not in the form of a plaque, but of a light cloud at the water-air boundary, and only in old cultures did this cloud drop down and form a diffuse grayish zone in the water. The distinct plaque that was so typical of the unpurified culture was not formed. However, when simultaneous seeding of the spirillum culture and a culture of the associated organism No. 3 was made, a distinct and typical plaque was again formed.

An experiment was set up in cylinders with seedings of the spirillum culture, of the associated organism No.3, and of both together. Control cylinders were set up in parallel with seedings of a killed suspension of spirilla and associated organism.

Cylinders were kept in the dark at 19-20 deg for a month. Sulfates were determined gravimetrically. The results are given in Table 4.

As seen from the experiment, the presence of the associated organism sharply stimulates sulfate formation by the spirillum culture, although the associated organism itself does not form sulfates and in some cases, as in the present experiment, sulfate consumption even occurs.

The spirilla are also able to form sulfates when grown in cylinders, in pure culture—but much less was formed than in the presence of the associated organism.

In all probability, symbiotic relations exist between the spirilla and associated organism and possibly the role of accompanying forms consists, in particular, in providing the spirilla with vitamins. At the same time the associated organism itself, as already pointed out, is not capable of autotrophic CO_2 assimilation and probably belongs to the oligocarbophil microorganisms since it can be retained and can grow, although with difficulty, in test tubes containing van Niel medium or distilled water where only agar (the agar used was carefully washed) or the air can serve as a source of organic compounds. The associated organism can obtain such amounts of organic material from the spirilla in symbiotic relationship.

Thus, the study of organisms in natural association gives a conception of their behavior in nature. At the same time a detailed knowledge of the properties of the individual organisms in necessary. For example, the fact that spirilla are heterotrophes and yet can shift under certain conditions to an autotrophic mode of life is, in all probability, closely related to the

Table 3. Growth of Spirillum Cultures Passed Through Organic Media to Liquid Inorganic van Niel Medium

Expt. variant		CO_2 in mg/liter		H_2S in mg/liter		H_2SO_4 in mg/liter	
		determined	consumed	determined	oxidized	determined	formed
	Killed cells (control)	515		68.3		Not	
Living cells (expt.)	Culture from MPA	498	17	65.6	2.7	determined	
	Culture from peptone	498	17	65.3	3.0		
	Killed cells (control)	632.6		108.4		55.6	
Living cells (expt.)	Culture from wort	603.0	29.6	102.8	5.6	36.0	19.5

Table 4. Sulfate Formation during Growth of Spirilla, of Associated Organism No. 3, and of a Mixture of Both in Cylinders Containing Water and Agarized van Niel Medium at the Bottom

Expt. variant	H_2SO_4 in mg/liter	
	determined	formed (+) or consumed (−)
Control	35.0	
Spirilla	42.9	+ 7.9
Associated organism	30.2	− 4.8
Mixture (spirilla + associated organism)	57.6	+22.6

seasonal changes in content of organic matter in the water. At periods when the organic content of the water has been impoverished through the activity of various groups of heterotrophic organisms and at the same time enriched with H_2S, the spirilla remain active owing to their autotrophic properties.

SUMMARY

1. A pure spirillum culture has been isolated from a natural association of microorganisms growing in the form of a plaque in test tubes containing agarized van Niel medium under a water layer.

2. The spirilla have the capacity for an autotrophic mode of life, consume CO_2 during oxidation of H_2S to sulfate, but require the presence of vitamins.

3. The spirilla grow well as typical heterotrophs, but retain the capacity for an autotrophic mode of life after cultivation on organic media.

4. It is suggested that there exist in nature symbiotic relations between spirilla and accompanying organisms, and a connection between seasonal fluctuations in organic content of water and the capacity of the spirilla to shift from a heterotrophic to an autotrophic mode of life.

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MORPHOLOGY OF NITROSOMONAS EUROPAEA

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The arrangement and number of flagella in bacteria are considered to be a typical and persistent characteristic and are utilized in the present-day classification of microorganisms.

Earlier determinations of number and arrangement of flagella in bacteria were made by examining preparations fixed and stained by special methods in the ordinary microscope. The magnification of a thousand times did not allow a definition of the details of structure and attachment of flagella, since the diameter of flagella does not exceed 30-35 mμ. In making preparations, the flagella were frequently torn off or agglutinated. Use of the electron microscope offered new opportunities for detailed study of bacterial morphology. The organs of motion in Agrobacterium, Rhizobium, and Chromobacterium have been studied in detail by Conn and Elrod (1947). Van Iterson (1949) studied the structure and attachment of flagella in a number of Pseudomonas species. Hampp, Scott, and Wyckoff (1948) made electron micrographs of flagella in certain spirochetes, including Treponema pallidum. The flagella of this organism could not be detected in the ordinary microscope.

It had been claimed earlier that the majority of motile forms of Azotobacter were monotrichs. Hoffer (1944) found in studying a number of Azotobacter species that they were all peritrichs and have a large number of flagella.

Khudyakova (1950) studied 11 cultures of different species of Azotobacter with the electron microscope and also concluded that they were all peritrichs, the type of flagella arrangement being different in different species.

The morphology of Nitrosomonas europaea has not been studied in detail since Vinogradskii. In a study published in 1892, Vinogradskii describes the shape of the cells, presence of only one flagellum, and alternation of motile and nonmotile stages. Kingma Boltjes (1935), Imshenetskii (1945), Lees (1953), Ul'yanova (1955), and other authors cite data on the morphology of Nitrosomonas in their studies, but do not deal with the growth cycle of this microorganism.

Present-day methods of microscopic observation permit more detailed study of the morphology of Nitrosomonas without treating the cells with fixing agents, dyes, and mordants that may distort the shape and size.

The strain of N. europaea we studied in the ordinary optical microscope did not differ in either size or shape of cells from the microorganism described by Vinogradskii. N. europaea was cultivated on ordinary Vinogradskii medium containing calcium carbonate at 26 deg. After 5-8 days of growth a large number of motile cells can be seen in the culture.

In order to study Nitrosomonas in the electron microscope, preparations were made by the dialysis method; several drops of culture were placed on a film of collodion, which had been dissolved in amyl acetate, floating on the surface of distilled water. After 12 hours the fluid from the dialyzed drops was transferred to grids and the preparations were as usual, dried and dusted with chrome.

It was found that Nitrosomonas possesses not just one flagellum, as described by Vinogradskii, but a tuft of flagella (4-8) attached to one of the cell poles (Fig. 1). Sites of attachment of flagella can be seen in Fig. 2. During cell division only one of the cells has flagella, the other is usually without them (Fig. 3).

Apparently in staining flagella by the method of Loeffler, employed by Vinogradskii, the flagella are agglutinated into one strand. Such agglutination or twisting of flagella can be seen also under the electron microscope (Fig. 4).

Nitrosomonas cells divide after 8-10 days of growth, but do not separate, and form the zooglea-like masses described by Vinogradskii. Some cells of the zooglea do not lose their flagella immediately (Fig. 5). Around zooglea composed of a large number of cells can be seen flagella that have separated from cells and ones that have not (Fig. 6).

The tuft of flagella in living, unfixed cells can be seen with the anoptral microscope (Fig. 7).

The growth cycle of Nitrosomonas, which we followed, agrees with the data of Vinogradskii, but we were able to subdivide it into four stages, whereas Vinogradskii mentions only two.

I. Cells adhere to sediment or bottom of vessel and are nonmotile (3-4 days).

II. Majority of cells with flagella are actively motile, almost none adhere to particles of sediment (5-8 days).

III. Cells at division stage, some shed their flagella (8-10 days).

IV. Cells without flagella form zooglea-like masses in the sediment (10-12 days).

Start of the motile stage usually coincides with the most active nitrite accumulation. Transition to the zooglea stage occurs at the time of disappearance of all the ammonia in the medium.

The time intervals of the transition from one stage to another may shift, depending on the amount of seed material and composition of the medium, but the sequence of the stages is usually retained. Cell division is especially active toward the end of the motile stage. Cells are separated by a septum (Fig. 8) and, after dividing, one side remains depressed for a time.

I am grateful to R. M. Legunkova and V. M. Novogorodtseva for assistance in carrying out this study.

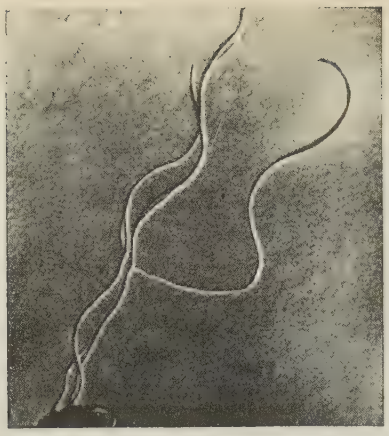

Fig. 1. Part of a Nitrosomonas cell with three flagella. Electron microscope. Mag. 10^4×.

Fig. 2. Nitrosomonas cell with flagella. Electron microscope. Mag. $5 \cdot 10^4$×.

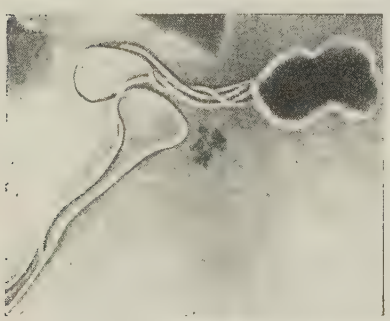

Fig. 3. Dividing Nitrosomonas cell with flagella at one pole. Electron microscope. Mag. 10^4×.

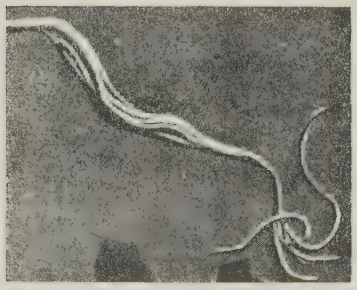

Fig. 4. Nitrosomonas with tuft of flagella entwined in one strand. Electron microscope. Mag. 10^4×.

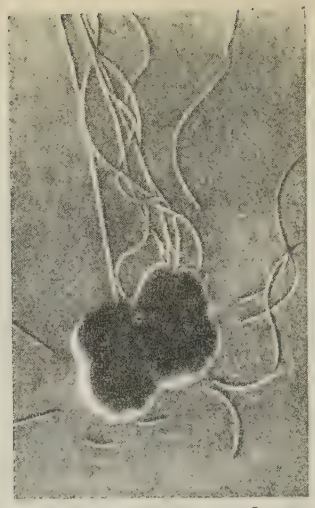

Fig. 5. Beginning stage of zooglea formation. Cells have not yet lost flagella. Electron microscope. Mag. 10^4×.

Fig. 6. Mass of Nitrosomonas cells, flagella around zooglea. Electron microscope. Mag. 10^4×.

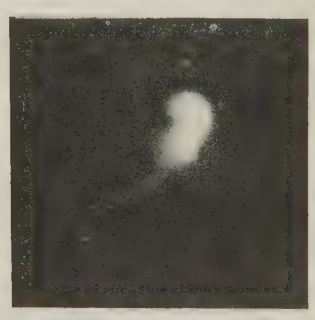

Fig. 7. Dividing cell with tuft of flagella at one pole. Anoptral microscope. Mag. $4 \cdot 10^3$×.

Fig. 8. Divided Nitrosomonas cells. Anoptral microscope. Mag. $4 \cdot 10^3$×.

SUMMARY

Electron micrographs showed that Nitrosomonas europaea is a lophotrich, and not a monotrich as claimed earlier.

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THE LIFE CYCLE AND NUCLEAR APPARATUS IN HYPHOMICROBIUM VULGARE STUTZ. AND HARTLEB

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Henrici and Johnson (1935), studying growth plates in fresh waters, found a unique group of attached organisms that they classified in the independent order Caulobacteriales, class Schizomyceae. They considered that Hyphomicrobium vulgare, described by Stutzer and Hartleb at the end of the last century, would be typical of one of the families of this order, Pasteuriaceae, if multiplication of this organism by budding could be demonstrated. Such results were obtained by Kingma Boltjes (1936), and later confirmed by Mevius (1953). In the seventh edition of Bergey's Manual (1957), budding organisms were taken out of Caulobacteriales and put in the independent order Hyphomicrobiales Douglas. However, the multiplication by polar budding described in Hyphomicrobium caused Stanier and van Niel (1941), to doubt whether Caulobacteriales should be classified under Schizomyceae.

Hyphomicrobium also occupies an intermediate position with respect to its physiology between the two basic physiological groups, autotrophs and heterotrophs. Hyphomicrobium oxidizes simple organic acids and alcohols to carbon dioxide. It uses carbon dioxide as a carbon source to form its body substance.

The present paper describes the life cycle and transformations of nuclear material in Hyphomicrobium vulgare.

MATERIAL AND METHODS

Hyphomicrobium is widely prevalent in various natural sites. We have found it in specimens of cultivated soil from Europe, Asia and Africa, it is widely prevalent in various fresh waters, it is encountered in decomposing rocks, large numbers are present in activated sludge, and it frequently contaminates laboratory cultures of algae. It was present in almost all specimens in which an agent of the second phase of nitrification was found.

The following three basic methods were used to cultivate Hyphomicrobium:

I. Growth in Erlenmeyer flasks in the liquid inorganic medium of Mevius ($NaNO_3$—1 g; K_2HPO_4—1 g; NaCl—0.5 g; $MgSO_4 \cdot 7H_2O$—0.5 g; 1 liter tap water). To a 100-ml flask was added 20 ml of medium and after sterilization several drops of methanol were added. On incubation at 28 deg growth appeared in 3–5 days in the form of a delicate film along the vessel walls and then covered the whole surface; in a week, a heavy dry pellicle had formed (Fig. 1).

II. Individual colonies can be obtained on the same inorganic medium by adding 2% carefully washed agar. The inoculum was added with a spatula to the agar surface, the plate was inverted and a drop of methanol applied to the lid. White colonies of Hyphomicrobium appeared in 4-5 days after incubation at 28 deg and then increased in size in the course of several months, reaching a diameter of 1-2 mm. The colonies (Fig. 2) have a typical concentric structure, the border is delicately fringed.

Fig. 1. Hyphomicrobium pellicle on Mevius medium containing methanol.

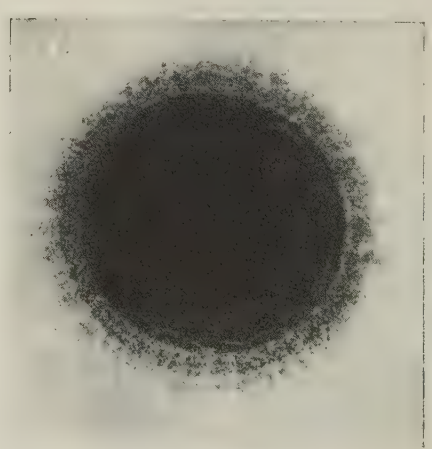

Fig. 2. Hyphomicrobium colony on agar in methanol vapor, 2 months.

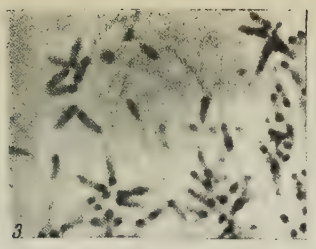

Fig. 3. Hyphomicrobium cells grown on liquid inorganic medium in methanol vapor. The terminally placed nuclei are seen clearly in the cells. Phase contrast.

Fig. 4. Young Hyphomicrobium rosettes in liquid inorganic medium in methanol vapor. Nuclei have converged to the rosette center, in some cells the nuclei have divided. In the central rosette of 6 cells the hyphae have just begun to elongate, in other rosettes buds have already formed. Phase contrast.

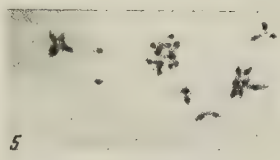

Fig. 5. Same as Figure 4, but stained with fuchsin after hydrolysis. Cell aggregation into young rosettes of 2-3 cells is seen well. Some hyphae branch.

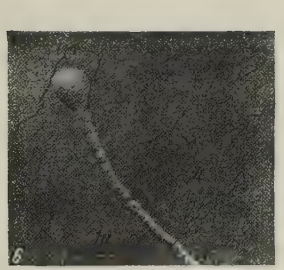

Fig. 6. Young bud at end of hypha. Electron microscope.

Fig. 7. Bud shortly before separation. A very fine flagellum is seen, attached subterminally to the broad end of the cell, and the site of hypha breaking. Electron microscope.

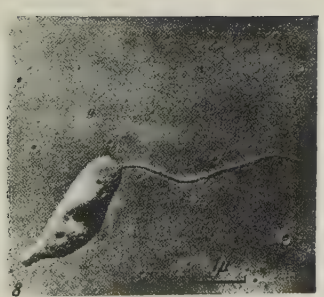

Fig. 8. Separated motile Hyphomicrobium bud. At the end opposite the flagellum the cell has a fragment of the hypha that connected it to the mother cell. The rounded nucleus is seen within the cell. Electron microscope.

Fig. 9. Young Hyphomicrobium rosette. Cells have shed flagella, hyphae have just begun to elongate. Nuclei are seen well within the cells. Electron microscope.

III. For cytological study, it was more convenient to grow Hyphomicrobium in a tall liquid layer in tubes. Tubes containing 10 ml of medium, seeded with Hyphomicrobium culture, were placed in a carton that also contained an open tube of methanol. In 4-5 days the Hyphomicrobium gave even growth throughout the layer of medium and reduced nitrate to nitrite. Only a very delicate film appeared on the surface. Under these conditions few masking substances were formed and the structural details of the living untreated cell could be distinguished. Furthermore, the microcolonies—"rosettes"—of Hyphomicrobium, had a more regular shape here.

Certain Hyphomicrobium strains also grow on potato agar or minced potato for several passages, but then they die. Growth is observed in yeast water. Hyphomicrobium grows on silica gel by utilizing organic substances in the atmosphere.

In the present study, a strain of Hyphomicrobium isolated from cultivated soil near Moscow was used. A mass culture was obtained by seeding a clump of earth on Medium I. A pure culture was isolated by transfers from individual colonies to solid medium (II). A pure culture was obtained after several passages. Purity was checked microscopically and by seedings on meat-peptone agar, meat-peptone broth, and wort agar. On these media, the pure cultures gave no growth.

MORPHOLOGY

The shape of Hyphomicrobium cells varies considerably, depending on cultural conditions. The description given below refers to growth on liquid inorganic medium in the presence of methanol vapor (III).

Figure 3 gives a picture of the nature of Hyphomicrobium growth. The elongated spindle-shaped cells have an average size of $0.5 \times 2\,\mu$. At one of the ends of the cell axis there is an outgrowth, a hypha $0.2\,\mu$ in diameter. Its length fluctuates greatly, from several microns on potato agar to hundreds of microns on silica gel. In the latter case the hyphae frequently exhibit true branching. In growth on Mevius medium containing methanol, the hyphae are $10\text{-}20\mu$ long and branch much more rarely (Fig. 5). On staining with Ziehl's fuchsin and in electron photographs there can be discerned within the hypha a zoned structure consisting of more and less dense sections. The hypha has a membrane in common with the cell (Fig. 9).

Hyphomicrobium cells are gram-negative and stain poorly with methylene blue. Like Nitrobacter cells, they are almost invisible in the light microscope.

A nucleus can be detected within the cell by various methods that is very similar in shape to the nucleus of myxobacteria. The Hyphomicrobium nucleus is most frequently round, much more rarely it has the form of a transverse band bisecting the middle third of the cell.

The following methods were used to detect the nucleus: the Feulgen reaction, staining according to Peshkov, and staining with methyl green. Good results were obtained by staining with basic fuchsin in 1/10,000 dilution after 7-minute hydrolysis at 60 deg in 1 N HCl (Fig. 5). This method, however, should only be used in old cultures grown on media rich in organic compounds; in a number of cases there is no need of hydrolysis. The best method is phase-contrast microscopy of nuclei in living Hyphomicrobium cells grown in a tall layer of liquid in tubes. By means of this method it was possible to determine the transformations in the nuclear apparatus of Hyphomicrobium by observing a living culture.

All methods of observing the nucleus give concordant results (see Fig. 3, 4, and 5).

Budding in Hyphomicrobium has been twice photographed under visual control (Kingma Boltjes, 1936, and Mevius, 1953). Our observations gave similar results, hence the following description of the life cycle of Hyphomicrobium is illustrated with electron micrographs permitting the determination of some additional details. The electron microscopy was conducted in collaboration with R. Legunkova. The following stages can be distinguished in the budding of Hyphomicrobium. First, a small swelling appears at the end of a hypha (Fig. 6), and it gradually grows to reach the size of the mother cell.

The mature bud has a conical shape with the broad end outward. Prior to separation the bud begins to wave abruptly from side to side and breaks the hypha, as is seen well in the electron micrograph (Fig. 7). At this time, a very fine flagellum can be discerned in the bud, attached at the side of the broad end of the cell.

The separated cell swims away, carrying with it a short segment of hypha. The flagellum in Hyphomicrobium is relatively short, 3–4 , but the cell moves quite rapidly. The nucleus is also seen well in the photograph (Fig. 8).

In observing the living culture in liquid inorganic medium, we were able to determine the further fate of the cell. As seen in Figs. 3, 4, and 9, Hyphomicrobium cells are often arranged in rosettes, turning their hyphae outward. A motile cell swims up to such a rosette and fastens with its broad end to the center. It is more convenient to watch the process in single cells. It may be noted that a motile cell usually fastens to a nonmotile cell whose hypha has just begun to elongate. It is seen in Figs. 3 and 4 that between cells an isthmus is formed. Thus the cells of one rosette are joined together.

Phase-contrast photographs of a living culture enable one to picture the behavior of the nucleus in the process described. The nucleus is seen in the bud from the very beginning of its existence (Fig. 4). In the growing daughter cell it is located at the enlarged end of the cell, turned outward. The nucleus continues to occupy this position in the separated bud (Fig. 8). At the time of rosette formation the nuclei of both cells are located at the cell ends, free of hyphae, and with formation of the connecting-piece between cells they converge (Fig. 4). This position of the nucleus is retained in young rosettes. In older rosettes the nuclei are divergent and occupy a site at the hyphal base.

Dividing nuclei are observed in Hyphomicrobium at different stages of cell growth although, as a rule, division of the nucleus precedes the formation of a new bud. As the bud grows the second nucleus, which has moved to the hyphal base, disappears from the cell.

The results of the observations made permit the formation of a conception of the life cycle of Hyphomi-

crobium, which is depicted in a diagram (Fig. 10).

In examining the life cycle of Hyphomicrobium, two questions arise that require experimental solution.

First, the mechanism of transmission of nuclear substance along the hypha is not clear. Possibly the zoned structure in the hypha, observable in the electron microscope and when treated with fuchsin, corresponds to this process.

Second, it is not clear whether nuclear contact is made through the narrow connecting-piece between cells, i.e., whether the process of rosette formation can be considered to be associated with karyogamy.

These questions are difficult to answer definitely without a study of ultrathin sections.

Fig. 10. A diagram of multiplication and rosette formation in Hyphomicrobium vulgare.

DISCUSSION OF RESULTS

The method of reproduction and the cell structure of Hyphomicrobium distinguish it sharply from the true bacteria. It may be suggested that Hyphomicrobium is a transitional form between bacteria and fungi, probably the Mycochytridiales. Hyphomicrobium, however, differs from the latter not only by the fact that its physiology, method of nutrition, and dimensions are similar to those of bacteria. Despite the treatment of a large amount of material, we could not detect chitin in the membrane of Hyphomicrobium.

In classifying Hyphomicrobium, it would be more proper to examine its relation to other related organisms and then to determine the position of the entire group.

The same type of reproduction as in Hyphomicrobium occurs in the nonsulfur photosynthesizing microorganism, Rhodomicrobium van nielii, whose nuclear apparatus is entirely similar to the Hyphomicrobium nucleus (Murray and Douglas, 1950). Here, however, no motile stages were found, although some spherical buds did separate from the dendritic colony.

Nor does Gallionella planctonica (Blastocaulis) have motile stages, the life cycle of which was described by Guseva (1955). The spherical buds, after separation, do not have independent movement; they either are carried by a water current or slide to the base of pedicles, forming a star-shaped colony. Finally, Caulobacter, outwardly very similar to Hyphomicrobium and encountered in cultures of it, reproduces by segmentation, as does Gallionella ferruginea.

Nitrobacter and Pasteuria, which multiply by polar budding, should also be mentioned.

Thus Hyphomicrobium is related to bacteria through an entire series of transitional forms. Since reproduction by budding is encountered in bacteria as a non-typical process, Hyphomicrobium and related organisms are rightly regarded with the Schizomyceae, as the independent order Hyphomicrobiales Douglas, the distinguishing feature of this order being the characteristic structure of the nuclear apparatus.

SUMMARY

1. Hyphomicrobium vulgare is widely prevalent in nature. It is readily isolated in pure culture by transferring colonies grown on agar in the presence of methanol vapor.

2. The sole method of reproduction in Hyphomicrobium is by bud formation at the hyphal tip. The separated bud is motile owing to the presence of a subterminal flagellum. It also has a rudimentary hypha. The motile cell participates in rosette formation.

3. Hyphomicrobium has a nucleus of the myxobacterial type. Nuclear division as a rule precedes bud formation.

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EFFECT OF NUTRITIONAL CONDITIONS ON SURFACE STRUCTURE OF ACTINOMYCETE SPORES

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Actinomycete conidia were first studied in the electron microscope by Kriss et al. (1945). The strains employed by these workers had spores with a smooth surface. Subsequently Flaig et al. (1952; 1954/1955) noted that the conidia in certain actinomycetes may have a spiny, hairy, or warty surface.

The possibility of employing conidia morphology as a diagnostic feature in classifying actinomycetes has been discussed by many workers. Some, as for example Krasil'nikov (1949), with data based on light microscopy took into account only the general spore shape. Others, as for example Flaig et al. (1944/1955), Baldacci et al. (1955), Kutzner (1956), Ettlinger et al. (1958), took into account not only the general spore shape but also the type of surface structure revealed by the electron microscope.

Flaig et al. (1954/1955) reported that when certain actinomycetes are grown in the presence of organic nitrogen they form spores with spines. On employing nitrates in the medium as the sole nitrogen source, however, a decrease in spines and even their disappearance were observed.

Since the formation of conidia with a spine-covered surface may be regarded as a potential criterion for classifying actinomycetes, the present study was undertaken to test the stability of this feature under various nutritional conditions.

MATERIALS AND METHODS

The strains of actinomycetes studied were obtained from Professor N. A. Krasil'nikov's collection and they have been designated in the text by the terms he employed. It should be noted that the terms Actinomyces and Streptomyces are used synonymously in the present paper.

We employed 29 media. The final pH of these media varied in the range 6-7, except medium IX which was more acid (pH 5.0).

24 media were prepared from the basic medium by adding various supplements.

Composition of the basic medium: $Fe_2(SO_4)_3 \cdot 9H_2O$—10 mg; $ZnSO_4$—10 mg; $MgSO_4 \cdot 7H_2O$—0.1—0.5 g; K_2HPO_4—1 g; agar—15 g; distilled water—1 liter. (Media I-X were prepared with 100 mg $MgSO_4 \cdot 7H_2O$. Media XI-XXIV contained 500 mg $MgSO_4 \cdot 7H_2O$.)

Supplementation of basic medium: I, pressed beer yeast—10 g; glucose—5 g; II, asparagine—5 g; glucose—5 g; III, glutamic acid—10 g; glucose—5 g; IV, NH_4NO_3—5 g; glucose—5 g; V, $(NH_4)_2SO_4$—5 g; glucose—5 g; VI, glutamic acid—15 g; VII, aspartic acid—10 g; VIII, aspartic acid—10 g; glucose—5 g; IX, asparagine—5 g; glucose—5 g; $CaCl_2$—15 g; X, asparagine—5 g; glucose—5 g; NaCl—1.5 g; XI, $NaNO_3$—2 g; sucrose—30 g; XII, NH_4NO_3—1 g; sucrose—30 g; XIII, $(NH_4)_2SO_4$—1.5 g; sucrose—30g; XIV, $NaNO_3$—2g; maltose—30 g; XV, $NaNO_3$—2 g; glycerol—30 g; XVI, $NaNO_3$—2 g; glucose—30 g; XVII, $NaNO_3$—2 g; fructose—30 g; XVIII, $NaNO_3$—2 g; starch—30 g; XIX, $NaNO_3$—2 g; dextrin—30 g; XX, $NaNO_3$—2 g; xylose—30 g; XXI, $NaNO_3$—2 g; arabinose—30g; XXII, $NaNO_3$—2g; lactic acid—30 g; XXIII, $NaNO_3$—2 g; malic acid—30 g; XXIV, Czapek agar: $NaNO_3$—2 g; KCl—0.5 g; sucrose—30 g; $ZnSO_4$ was not added. Other media employed were starch-ammonia agar, wort agar, potato agar, fish agar, peptone-corn agar.

Actinomycetes grown on medium I were seeded on agar slants containing the various media.

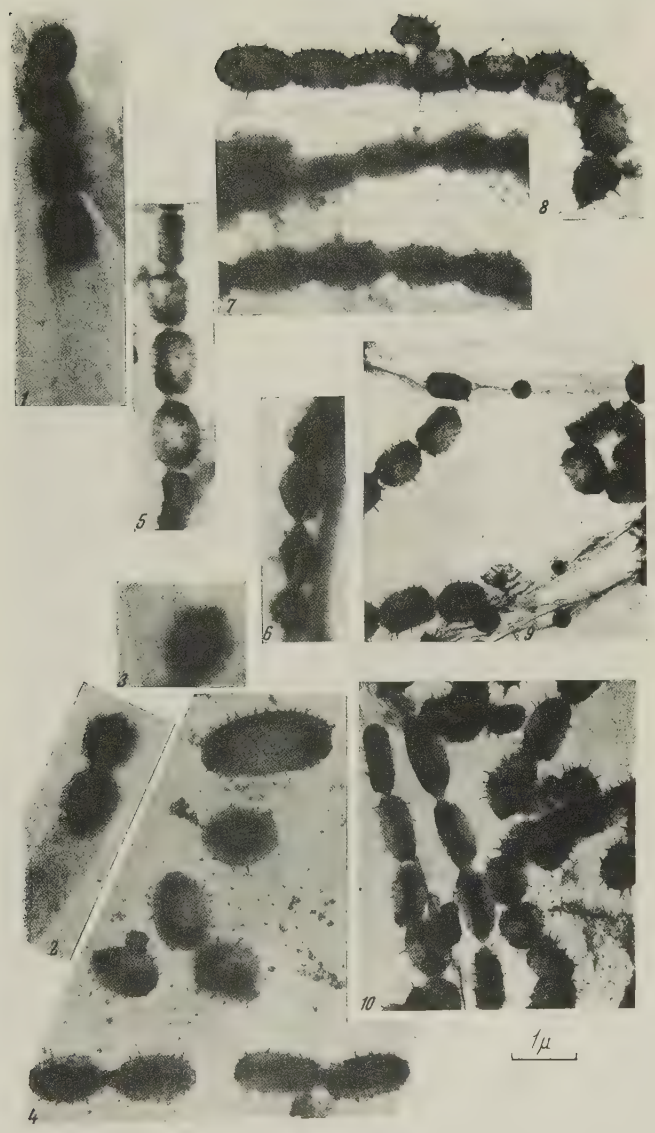
After incubation at 28-30 deg for 3-30 days, a drop of distilled water was applied to the surface growth in the culture and moved over the surface. When spore formation was abundant, the drop of water was covered with spores. Spore material obtained in this way was placed on an electron microscope grid covered with a formvar film. This method of making preparations gave a clear background for electron microscopy. If spore formation was not abundant, spore material was carefully scraped with a loop from the culture surface, suspended in a drop of water, and placed on the electron microscope grid. Preparations were dried in air and examined in electron microscope UEM-100 at 60 kv. Preparations were also made for the light microscope from the same drop of water containing spore material, fixed in the flame of a burner, and stained with methylene blue.

DISCUSSION OF EXPERIMENTAL DATA

Of 42 actinomycete strains grown on medium I and potato agar, the following had no spines on the spores: A. olivaceus strains 43, 44, 52, 1, 40, 45; A. fluorescens strains 16, 828; A. globisporus strains 2302, K-A-449, K-A-58; A. globisporus roseus strain 1849; A. levoris strain 3014; A. toxicus strains 2350, 2378; A. coelicolor strains 104, 206, 385, 78; A. aurantiacus strains 205, 2516, 1404, 1306, 1309; A. violaceus strain 71; A. violaceus chromogenes strain 2929; and A. vulgaris strain 1034.

The following cultures exhibited spiny outgrowths on spores: A. coelicolor 1047, 2078; A. violaceus—829, 1144, 2525, 2522, 2069, 2587, 117, 719; and A. viridochromogenes—93,94.

We consider it necessary to stress that the presence or absence of spines on spores in the cultures we studied provides no basis for far-reaching conclusions. On the one hand, the presence or absence of spines on spores may be the result of variability. Then too, it should be borne in mind, different authors employ the same name for different microorganisms or different



Figs. 1, 2, 3. Spores of A. viridochromogenes strain 94 after 3-day growth on asparagine medium II.
Fig. 4. Same after 10 days.
Fig. 5. Same after 16 days.
Figs. 6, 7. Spores of A. viridochromogenes strain 93 after 3-day growth on potato agar.
Fig. 8. Same after 15 days.
Figs. 9, 10. Same after 30 days.

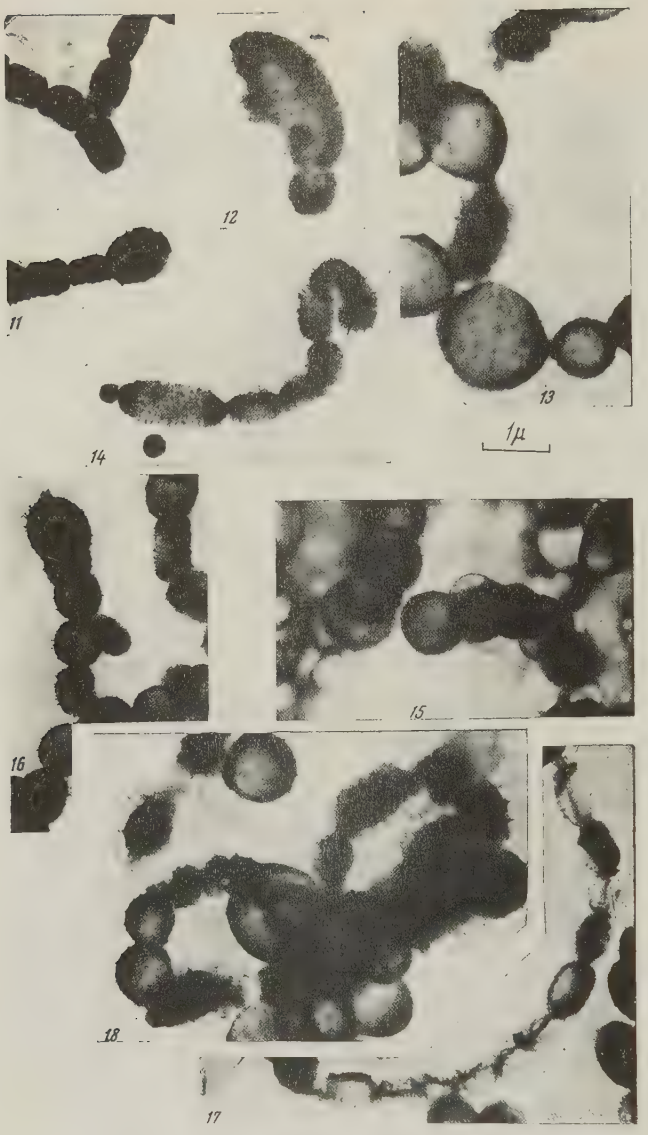


Fig. 11. Spores of A. viridochromogenes strain 94 after 14-day growth on medium containing lactic acid and $NaNO_3$ (medium XXII).

Fig. 12. Spores of A. viridochromogenes strain 93 after 30-day growth on potato agar.

Fig. 13. Spores of strain 94 after growth for 16 days on medium containing glucose and $(NH_4)_2SO_4$ (medium V).

Fig. 14. Same as Fig. 12.

Fig. 15. Spores of strain 94 after growth for 15 days on medium containing xylose and $NaNO_3$ (medium XX).

Figs. 16, 17. Spores of strain 94 after growth for 16 days on medium containing asparagine, glucose, and $CaCl_2$ (medium IX).

Fig. 18. Spores of strain 94 after growth for 16 days on medium containing glucose and NH_4NO_3 (medium IV).

Fig. 19. Spores of A. violaceus strain 829 after 4-day growth on potato agar.

Fig. 20. Spores of strain 829 after growth for 6 days on medium containing asparagine, glucose, and $CaCl_2$ (medium IX).

Fig. 21. Spores of A. violaceus strain 2525 after growth for 6 days on yeast medium containing glucose (medium I).

Fig. 22. Spores of strain 829 after growth for 20 days on medium containing maltose and $NaNO_3$ (medium XIV).

Fig. 23. Spores of strain 829 after growth for 20 days on medium containing starch and $NaNO_3$ (medium XVIII).

Fig. 24. Spores of A. violaceus strain 1144 after growth for 8 days on yeast medium containing glucose (medium I).

Fig. 25. Hyphae of A. violaceus strain 829 after growth for 11 days on medium containing asparagine and glucose (medium II).

Fig. 26. Sporophores of strain 829 after growth for 8 days on yeast medium containing glucose (medium I).

Fig. 27. Spores of strain 94 after growth for 16 days on medium containing glucose and NH_4NO_3 (medium IV).

Fig. 28. Spores of strain 93 after growth for 18 days on medium containing glucose and $NaNO_3$ (medium XVI).

Fig. 29. Spores of strain 94 after growth for 16 days on yeast medium containing glucose (medium I).

Fig. 30. Spores of strain 93 after growth for 18 days on medium containing glucose and $NaNO_3$ (medium XVI).

names for the same organism. Thus for example, in our opinion, the organism termed A. violaceus by Baldacci et al. (1955) differs from the A. violaceus culture used by us in this study. The latter is possibly to be identified with Streptomyces purpurescens (Ettlinger et al., 1958).

Four cultures were selected to investigate the effect of nutritional conditions on surface structure of actinomycete spores: A. violaceus strains 829 and 2525 and A. viridochromogenes strains 93 and 94.

The Course of Spine Formation in Actinomycete Spores with Time.

To determine the optimal incubation time in which spine formation on the spore surface occurs, one A. viridochromogenes strain (93) was grown on potato agar; the other (94) was grown on medium II. Preparations were made after 3, 15, and 30 days of incubation from strain 93; 3, 10, and 16 days from strain 94. Very young spores of the 3-day A. viridochromogenes 94 culture grown on medium II either had no spines (Fig. 1) or they were barely noticeable (Fig. 2 and 3). It can be seen in these same photographs that constrictions are formed between spores before the appearance of spines. The young spores of culture 93 grown on potato agar have short spines with a broad base (Fig. 6), the young spore chain being often enveloped by a slimy substance (Fig. 7).

With increase in length of incubation (beyond 10 days) the spores of these two strains acquired clearly delineated long thin spines (Figs. 4, 5, 8, 9, and 10). From these observations the conclusion can be reached that the spores are completed prior to spine formulation. The impression was gained that the process of spine formation occurred faster on potato agar than on glucose-asparagine.

Further observations were made on two-week old spore material.

General Morphology of the Four Cultures under Study.

The spores of A. viridochromogenes strains 93 and 94 were generally of elongated shape (Figs. 4, 5, 8, 9, 10, 28, 29), while those of A. violaceus strains 829 and 2525 were usually spherical (Figs. 19, 20, 22).

Spines were present only on spores. Hyphae of the organisms studied had no spines. Rudimentary spines were observed in one case on a sporophore in A. violaceus strain 829 (Fig. 26). Occasionally spines were observed on straight, rigid, opaque hyphae (Fig. 25). However, the probability is not ruled out that such hyphae represent a compact chain of spores that have not divided by constriction.

It should also be noted that the actinomycete hyphae sometimes contain very dense spherical or elongated bodies apparently having no relation to the spores (Figs. 9, 21). These spherical or rod-shaped bodies are often liberated by the mature culture in large quantities. They have been described previously by Baldacci et al. (1956) and Kriss et al. (1945).

The spores of an organism do not always react in the same way to drying, and some spores swell greatly under the vacuum conditions unavoidable in electron microscopy (Figs. 13, 15, 16, 18, 23, 27), with the result that the external layer separates from the inner surface of the spore. This phenomenon was observed especially often in preparations from actinomycetes grown on medium rich in inorganic nitrogen (Figs. 13, 15, 18, 23, 27). Sometimes empty spore membranes were seen in preparations, covered with spines and completely devoid of their inner contents, denser to electrons (Figs. 17 and 30). The impression is gained from these pictures that the spores of A. viridochromogenes have spines closely associated with the internal spore contents (Figs. 13, 18, 27), while in A. violaceus spores the spines are associated only with the outer spore membrane (Fig. 23).

Such observations suggest a difference in the mechanism of spine formation in these two species of actinomycetes. It is entirely possible that the spines, relatively uniform in size and shape, of A. viridochromogenes spores are indeed associated with the internal spore contents, while the spines in A. violaceus are generally not uniform in shape and are apparently formed as a result of extensions of some sort of slimy substance in the external spore layer (Fig. 24). This assumption can be confirmed more precisely by the method of ultrathin sections from spores of these organisms.

A similar difference in spine formation on spores from different species was observed by Flaig et al. (1954/1955), in relation to changes in nutritional conditions.

In addition to spores relatively uniform in size, larger bodies covered with spines were seen whose nature is not clear (Figs. 12 and 14). These bodies, most likely, are not the result of vacuum action, as was noted in other cases (Figs. 13 and 18). They are evidently giant spores. Other workers (Baldacci et al., 1956) have previously noted such structures.

Of particular interest are two smooth spores observed in an A. viridochromogenes strain 93 (Fig. 10) chain of spores having spines. The cause of this phenomenon is not entirely clear. Possibly the smooth spores were formed as a result of shedding the outer membrane with spines. This suggestion, however, is doubtful since we are assuming that the spines in this species are closely associated with the internal spore contents. Another explanation may be that the smooth spores arose as a result of mutation or segregation. This observation points to a possibility of employing spore surface morphology as a genetic marker.

Observations on Spores in Cultures Grown on Various Media.

The four actinomycetes were grown on the 29 media indicated (visual observations on growth of vegetative and aerial mycelium are given in the table).

The two A. viridochromogenes strains grew and formed spores on a greater number of media than the two A. violaceus strains.

Of the two A. viridochromogenes strains, strain 94 showed the best growth, while of the A. violaceus strains, strain 829 showed the poorest growth.

It was difficult to determine the presence of spines in spores on many media, as is indicated in the table by the sign (*). The spore chains were in some cases covered with some sort of substance giving them a rough appearance. In such cases they resembled im-

Type of Growth and Spore Morphology in Strains 829 and 2525 and Strains 93 and 94 on 29 Media

Medium	Strain No.	Mycel.growth vegetative	Mycel.growth aerial	Spores	Spines	Medium	Strain No.	Mycel.growth vegetative	Mycel.growth aerial	Spores	Spines
I	829	++	++	+	+	VIII	829	-+	-++	—	
	2525	-+	++	+	+		2525	++	-+	—	
	93	-+	++	+	+		93	++	-+	—	
	94	-+	++	+	+		94	-+	-+	+	*
II	829	++	++	+	*	IX	829	++	-++	+	+
	2525	-+	++	+	*		2525	++	-+	—	
	93	-++	++	+	*		93	-+	++	+	+
	94	-+	++	+	+		94	++	-++	+	+
III	829	++	++	—		X	829	+-	++	—	
	2525	++	++	—			2525	+—	+—	—	
	93	+-	++	—			93	++	++	—	
	94	++	++	+	*		94	+	+	—	
IV	829	+—	+—	—		XI	829	++	-+	+	+
	2525	+—	—				2525	-+—	—		
	93	+—	--				93	-++	-+-	+	+
	94	-+	+	+	+		94	-+-	-+	+	+
V	829	-+—	-+—	—		XII	829	+—	—		
	2525	-+—	—				2525	+—	—		
	93	+—	—				93	+—	-+—	—	
	94	-+	-+	-+	+		94	+—	+—	+	*
VI	829	-+—	+—	—		XIII	829	+—	—		
	2525	-+—	-+	—			2525	-+—	—		
	93	-+	+	—			93	+—	+—	—	
	94	-+-	++	+	*		94	-+—	-+—	+	*
VII	829	-+	-+	—		XIV	829	++	++	+	+
	2525	-+—	-+	—			2525	-+-	++	+	-+
	93	-+	-+	—			93	-+-	-+-	+	+
	94	-+	+	+	—		94	++	++	+	+

Medium	Strain No.	Mycel.growth vegetative	Mycel.growth aerial	Spores	Spines	Medium	Strain No.	Mycel.growth vegetative	Mycel.growth aerial	Spores	Spines
XV	829	-+	+	+	+	XVIII	829	+	+	++	++
	2525	-+-	-+-	-+	*		2525	+	+	++	++
	93	++	++	-+	+		93	+	+	++	++
	94	++	++	+	+		94	+	+	++	++
XVI	829	+	+	+	+	XIX	829	++	++	+	+
	2525	+	+	—			2525	++	++	+	+
	93	++	++	+	+		93	++	++	+	+
	94	++	++	+	+		94	++	++	+	+
XVII	829	—				XX	829	—			
	2525	—					2525	—			
	93	+—	++	+	+		93	—			
	94	+—	+	+	+		94	+—	+	+	+

Medium	Strain No.	Mycel. growth: vegetative	Mycel. growth: aerial	Spores	Spines	Medium	Strain No.	Mycel. growth: vegetative	Mycel. growth: aerial	Spores	Spines
XXI	829	--				Wort agar	829	++	++	—	
	2525	—					2525	++	++	—	
	93	+---	+—	+	+		93	+-	+—	—	
	94	+--	+--	+	+		94	+-	+-	+	+
XXII	829	+	+	+	*	Potato agar	829	++	++	+	+
	2525	+—	+—	—			2525	++	++	+	*
	93	+	+	+	*		93	++	++	+	+
	94	++	++	+	*		94	+-	+-	+	+
XXIII	829	+—				Fish agar	829	++	++	—	
	2525	--					2525	+—	+—	—	
	93	—					93	++	++	+	+
	94	—					94	++	++	+	*
XXIV	829	++	++	—		Peptone-corn agar	829	++	++	—	
	2525	++	++	—			2525	+-	++	—	
	93	++	+-	+	+		93	++	++	+	*
	94	+-	+—	+	+		94	++	++	+	*
Starch-ammonia agar	829	++	++	—							
	2525	+—	+—	—							
	93	+-	+-	+	+						
	94	++	++	+	+						

++, +, +— } intensity of mycelium growth

(—) absence of growth of mycelium, spores and spines; (+) presence of spores and spines; (*) doubtful cases of spine formation.

mature spores (Fig. 7). In other cases spores with and without spines were seen in the preparations (Fig. 11). When the actinomycetes were grown on media containing pentose (media XX and XXI), the slimy substance got into the water used for collecting the spores and contaminated the electron microscope preparations (Fig. 15).

Despite the difficulties encountered in employing a large series of media, it became obvious that spine formation on spores is a relatively stable characteristic. Only on one medium where aspartic acid was the sole source of carbon and nitrogen and only in the case of the one A. viridochromogenes strain 94 were all the that spore formation was in general sparse on this medium.

As was noted previously, the spore shape varies somewhat on different media. Polymorphism of spores is noted particularly in actinomycetes grown on complex organic media; hence in studying the spore surface in actinomycetes, media should be employed that contain inorganic nitrogen, and maltose or glycerol as carbon source.

This work was performed in accordance with an exchange program between the Institute of Microbiology AN SSSR and the Institute of Microbiology, Rutgers University, USA.

SUMMARY

1. The surface structure of spores from 42 actinomycete cultures was studied with the electron microscope.

2. The process of spine formation was followed in spores from two strains of Actinomyces (Streptomyces) viridochromogenes.

It was found that first the cytoplasm of the spore-bearing hypha condenses, giving rise to well-formed spores. The sporophores become empty of cytoplasm. Spines are formed on the surface of spores as they mature.

3. A. viridochromogenes strains 93 and 94 and A. violaceus strains 829 and 2525, grown on media containing various carbon and nitrogen sources with varying ratio of carbon to nitrogen and varying salt concentration, had spores with spines on nearly all media. Spores with and without spines were formed on some media. On certain other media the spore spines were not distinct.

4. The mechanism of spine formation in A. violaceus spores is different from that in A. viridochromogenes spores.

5. The use of media containing maltose, glycerol, and an inorganic nitrogen source is recommended for study of the spore surface in actinomycetes, since reproducible results are more readily obtained on these media.

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ELECTRON MICROSCOPY OF SPORES OF VARIOUS ACTINOMYCETE SPECIES

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A rather restricted number of diagnostic features are utilized at the present time in classifying the actinomycetes. This circumstance has caused a number of authors, in order to classify species more precisely, to seek additional features, such as utilization of different carbon and nitrogen sources by actinomycetes, antagonist properties, serological criteria, phage specificity, etc.

Together with the studies in this direction (Krasil'nikov, 1949), the morphology of sporophores and spores has been investigated in greater detail.

There are data on the nature of the spore surface in 28 actinomycete species in the studies of Kriss (1945), Carvajal, (1946), Bringmann (1951), Kuster (1953), Rautenshtein et al. (1953), Flaig et al. (1952, 1955, 1958), Baldacci et al. (1955), Corbaz, Ettlinger, Hutter et al. (1955, 1956, 1957a,b,c,d, 1958), Enghusen (1955), Vernon (1955), and Nikitina (1956).

The most thorough study of spore morphology in actinomycetes was made by Flaig and Kutzner (1958). By no means have all species of actinomycetes been investigated from this point of view.

The purpose of the present study was to investigate spore morphology in 800 actinomycete cultures belonging to 100 different species with the electron microscope. Preparations were made with offprints of spores from actinomycetes grown on synthetic medium*. Spores were examined once, in most cases on the 7-10th day of actinomycete growth and only typical cultures of the various species were examined again.

As already pointed out in our article (1959), the structure of the spore membrane in actinomycetes is related in a number of cases to the color of the aerial mycelium and is of different diagnostic significance in different groups of actinomycetes.

We present below, factual data on species whose basic description is given in the book by Gauze.

Actinomycetes with Straw-Colored, Greenish Yellow, Grayish Yellow, and White Aerial Mycelium.

Actinomycetes with aerial mycelium of this color, belonging to the series Helvolus, Albus, and Albosporeus (23 species), had a spore membrane with a smooth surface.

In most of the species of this series the spore shape varied greatly. Basically they were oval and oblong (Figs. 1,2). Certain species were encountered, however, with a preponderance of spores of specific shape. Thus, most of the strains of A. cremeus species have rod-shaped spores (Fig. 3); A. rubiginosohelvolus has cylindrical ones. Not a single species of the series Helvolus, Albus, and Albosporeus had round spores.

Actinomycetes with Pink and Lilac Aerial Mycelium.

All the actinomycete species studied in the series Lavendulae roseus, Fradiae, Fuscus, Ruber, and Roseoviolaceus, except two species, have smooth spores that are chiefly oval or oblong; spherical spores were not observed. Spore size varies not infrequently in strains of the same species, sometimes it is different even in the same strain; but there were often transitional forms between the extremes. No differences among species could be determined with respect to spore size and shape. Two species in this group, A. violascens and A. roseoviolaceus, have spiny spores (Fig. 4). It should be noted that A. violascens is distinguished from A. lavendulae by the basic color of the aerial mycelium (lavender-violet and pinkish or grayish lilac, respectively), as is A. roseoviolaceus (pink aerial mycelium) from A. violaceus (gray aerial mycelium). The demarcation made earlier between these species on the basis of aerial mycelium color has been substantiated by a difference in spore structure: A. lavendulae—smooth, A. violascens—spiny; A. violaceus—smooth, A. roseoviolaceus—spiny.

However, such cases are by no means always observed, since spores in the great majority of species in this group are smooth and cannot be utilized to distinguish species.

Actinomycetes with Bluish and Bluish Green Aerial Mycelium.

Actinomycete spores in the Coerulescens series are distinguished from those in the preceding series by the presence of various outgrowths on the membrane.

Most species in this series have spiny spores of average dimensions, not exceeding the spore width (Fig. 5). Such spores were observed in the species A. coerulescens, A. coerulescens var. longisporus, A. viridochromogenes, A. coeruleofuscus, A. bicoler, A. coeruleorubidus, and A. chartreusis.

Among the cultures that we classified under the species A. chartreusis, there is one strain with large spines whose length is not infrequently equal to the diameter of the spore itself (Fig. 6).

Strains of A. glaucescens and A. glaucescens v. badious species have spores with numerous hairy growths not exceeding the spore length.

It should be noted that spores with smooth surface were found among the strains of the A. coerulescens seeded on oat-infusion agar. These strains did not differ in cultural characteristics from those spiny spores.

As is evident from the data presented, the actinomycete species with bluish and bluish green aerial mycelium have, as a rule, spiny spores or spores with hair-like growths (except 6 strains of A. coerulescens species).

*For medium composition see book by Gauze et al., Problems in Classification of Actinomycete Antagonists (1956) p. 22.

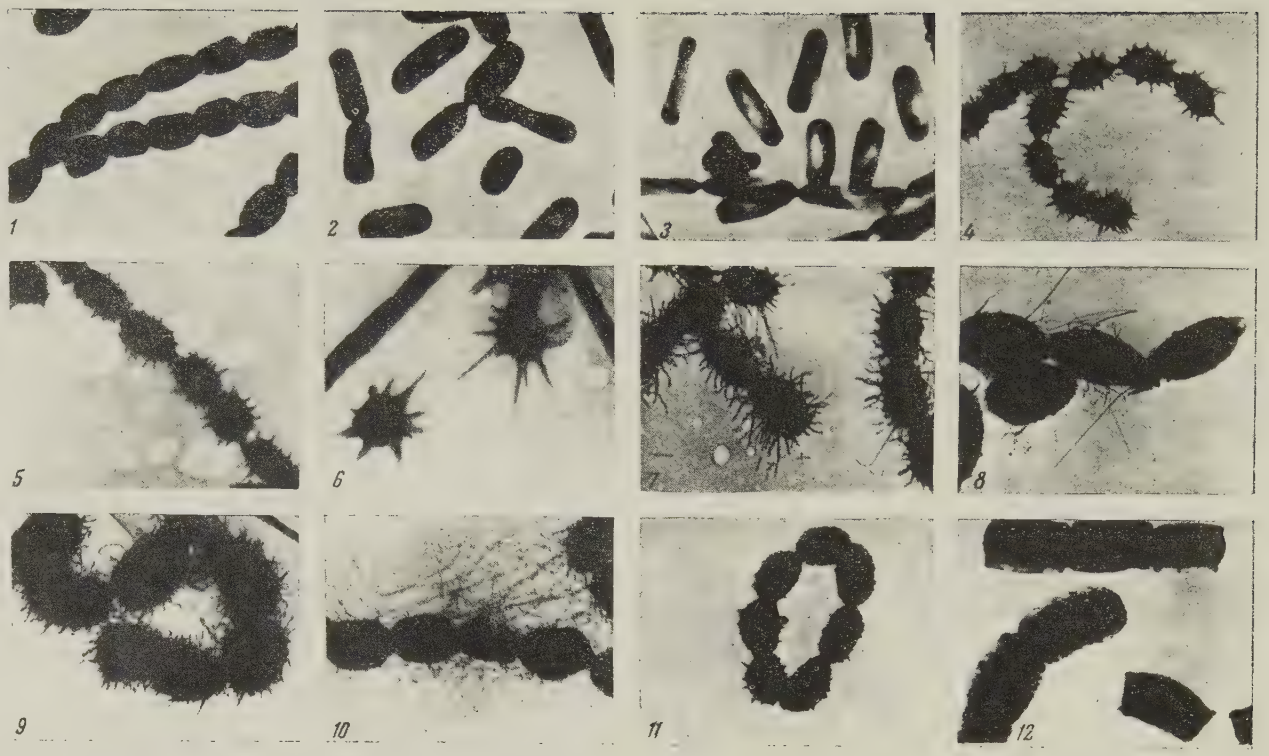

Fig. 1. A. globisporus var. flavofuscus.
Fig. 2. A. mirabilis.
Fig. 3. A. cremeus.
Fig. 4. A. roseoviolaceus.
Fig. 5. A. coeruleofuscus.
Fig. 6. A. chartreusis.
Fig. 7. A. griseus No. 605.
Fig. 8. A. acrimycini.
Fig. 9. A. griseostramineus.
Fig. 10. A. variabilis var. roseus.
Fig. 11. A. variabilis.
Fig. 12. A. aurantiogriseus.

Actinomycetes with Gray Aerial Mycelium.

Whereas a certain relation was found between color of earial mycelium and morphology of spore membrane in the actinomycete groups we examined previously, in the group with gray aerial mycelium all types of spore membrane are encountered: smooth, spiny, and hairy.

Spores of diverse morphology were observed in actinomycetes of the Griseus series with gray aerial mycelium and colorless substrate mycelium.

Cultures belonging to the species A. griseus Krainsky† mostly have spores with spiny or hair-like growths. The hairs are numerous, not exceeding the spore length (Fig. 7). The spines of spores with spiny growths are small, similar to the growths in A. variabilis. Cultures with smooth spores are also encountered in strains of this species.

An interesting feature is present in spores of the species A. acrimycini. They exhibited sparse hairlike growths exceeding the spore length (Fig. 8). In contrast to A. acrimycini, a variety of it, A. acrimycini var. globosus, had spores with numerous short outgrowths.

A. rochei and A. griseovariabilis strains have smooth spores.

Members of A. griseorubens species have very fine spines. A. rubiginosus strains were observed to have spine-like outgrowths on the spore membrane, resembling the hair-like type.

Spore morphology in actinomycetes of the Aureus series is just as diverse as in members of the Griseus series.

The actinomycete species A. aureofaciens, A. kurssanovii, A. flavochromogenes, A. antibioticus, A. flaveolus var. rectus have smooth spores. A. flavoviridis, A. flaveolus, and A. ambofaciens have spores with hair-like growths. A. griseostramineus spores, larger in size, also have hair-like growths on the membrane (Fig. 9).

Actinomycetes of the species A. flavochromogenes var. heliomycini have spores with hair-like growths intermediate between spiny and hair-like.

Strains of A. olivaceus species have smooth spores.

Cultures which we classified under A. graminearus had slight differences in cultural characteristics that did not permit subdivision into distinct varieties. The nonuniformity of this species was confirmed by the observations on spore morphology. Great diversity in spore morphology was observed in cultures of this species. Evidently A. graminearus, as well as the species A. griseus, A. flavochromogenes var. heliomycini, A. coerulescens, and others, is a miscellaneous group and can be subdivided in the future, as various strains are accumulated, into a number of species and varieties.

Thus, the Aureus series contains species both with smooth and hairlike and spiny spores.

Most species of the Chrysomallus series have a spore membrane with smooth surface. Of the species studied in this series, spores of two types, spiny and hairlike, were observed only in strains of the species A. malachiticus.

Three species in the Chromogenes series, A. cylindrosporus, A. fumosus, and A. chromofuscus have smooth spores, four others have various outgrowths on the membrane. Long hairlike growths were observed in A. variabilis var. roseolus (Figure 10). In most A. variabilis strains the spores are spiny (Fig. 11). Growths of this type, hair-like and spiny, were also observed in A. griseoincarnatus strains. A spore surface with fine spines is typical of members of A. chromogenes.

The great majority of species in the Violaceus series has smooth spores. A. aurantigriseus, which we classify at the present time in the Violaceus series, has a rarely encountered spore shape in the form of large cylinders (Fig. 12). Similar spores were noted earlier by Flaig and Kutzner (1958).

Of all the species studied in this series, outgrowths on spores were observed in only two: A. violaceoniger var. crystallomycini—spiny, and A. viridiviolaceus—hairlike.

It is of interest to note that species with greenish gray color of aerial mycelium most frequently have hairlike growths on the spore membrane (A. viridiviolaceus, A. variabilis var. roseolus, A. acrimycini, and others).

SUMMARY

1. An electron microscope study has been made of spore structure in 800 actinomycete cultures of different species described in the monograph by G. F. Gauze et al., Problems in Classification of Actinomycete Antagonists.

2. Three varieties of spiny outgrowths were observed: a) Fine spines; b) spines of intermediate size, not exceeding one-half the spore length; c) large spines equalling spore length.

3. Hairlike growths were of two types: a) Numerous short hairs, not exceeding spore length; b) long hairs equalling or surpassing spore length.

4. Factual data from electron microscopy of spores in individual species are presented.

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STUDY OF COLONY STRUCTURE IN ACTINOMYCES STREPTOMYCINI KRAS.

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Despite numerous investigations of colony morphology in actinomycetes, both natural strains and mutants, very few studies deal with microscopic structure and colony development on agar media (Lieske, 1921; Krasil'nikov, 1938, 1950; Knaysi, 1944; Erikson, 1947, 1948, 1949).

A careful cytological study of mycelium in Actinomyces coelicolor colonies was made by Bugrova (1949). However, she studied the mycelium only in preparations of crushed colonies, in smears, and consequently gives no data on the morphogenesis of actinomycete colonies, which is of much interest in connection with their classification and biology. The microtome section technique has scarcely been used for studies in this field. The present study is based on this method.

MATERIAL AND METHODS

The study was made with two streptomycin-producing cultures, Actinomyces streptomycini Kras. strains 58 and LS-3, obtained from the Museum of Cultures, All-Union Scientific Research Institute of Antibiotics (VNIIA).

The material investigated was colonies 2-25 days of age grown on the pea-infusion agar which is customarily employed to culture these strains in the Selection Laboratory of VNIIA.

Colonies were cut out of the agar, fixed in Carnoy's fluid or chrome-acetic-formol according to Navashin, passed through alcohols and xylol in the usual way, and embedded in paraffin. Small colonies 1-5 days of age were fixed whole, large colonies (5-25 days old) were cut in pieces. Microtome sections were 2, 3, and 5μ thick. Thickness of actinomycete hyphae varied around 0.5μ. Hence the best results were obtained with the thinnest sections.

Imprint preparations from colonies of varying age were also used in the study. They were stained with iron hematoxylin according to Haidenhein to determine general colony morphology, with gentian violet and methylene blue to determine degree of protoplasm basophilia, according to Feulgen to detect nuclear elements and their DNA content, and with methyl green and pyronine to determine content and distribution of RNA and DNA in hyphae.

Strain 58 was isolated from soil in N.A. Krasil'nikov's laboratory; it was obtained by stepwise selection in seedings of a natural culture, not subjected to the action of mutagenic factors, and can serve as a representative of natural cultures of Actinomyces streptomycini Kras.

Strain LS-3 was obtained from Waksman's collection. The two strains (58 and LS-3) are similar in basic cultural and morphological characteristics, physiological properties, and antibiotic activity. The antibiotic activity varied in the range 300-450 units/ml.

RESULTS

Colonies of the two strains on pea-infusion agar of greenish gray color sporulate well. The two-day old colonies from strains 58 and LS-3 are composed of two sharply different layers: A substrate layer composed of loosely disposed hyphae and a suprasubstrate layer consisting of densely intertwined hyphae (Fig. 1).

Mycelium of the substrate portion of the young colony (2 days) is made up of a network of intertwined hyphae, freely growing into the depth of the agar. The intense pyroninophilia in the protoplasm of these hyphae indicates its still high RNA content.

The suprasubstrate portion of the two-day colony consists of two layers: The mycelium adjacent to the agar surface is formed of a layer of hyphae growing horizontally (Fig. 1); above this layer a thick network of intertwined hyphae grow out from it; their free growing ends, forming the top of the colony, have a tendency to grow inward or parallel to the colony, where their source of nutrition is. For this reason, most of the surface is flat or knobby in the two-day colony (also in the 3-day colony of strain 58). However, even on the second day in some sections of the colony surface, there appear free growing ends of hyphae with a tendency to rise above the colony; this is the origin of the growth of hyphae in the aerial mycelium (Fig. 1).

On the third day in the substrate portion of the colony, the primary growth of mycelium forms an upper strong dense layer of hyphae. Their cytoplasm is more basophilic and nuclear elements give a stronger nuclear reaction than in hyphae of peripheral parts of the colonly up to 10-15 days of growth (Figs. 2 and 6:ПМ).

On the third day of growth, however, as the medium is exhausted and products of cell activity diffuse into the medium, growth of the basophilic hyphae of growth phase I in the substrate portion of the colony stops; it is succeeded by the appearance of loosely disposed hyphae of growth phase II, extending downward and sideways from the area of primary growth of the colony (Figs. 2 and 6).

Hyphae of growth phase II contain weakly basophilic cytoplasm and sparsely disposed nuclear elements and stain very weakly in the Feulgen reaction.

Strain 58 is distinguished from strain LS-3 by its particularly strong growth of substrate mycelium; greatly thickened phase II hyphae are localized in its lower region, with clearly differentiated nuclear elements (Fig. 5) staining weakly in the Feulgen reaction.

On the third day of growth, the hyphae in the lower part of the substrate mycelium, located under the colony center (area of primary expansion of colony), completely stop growing down into the medium, and grow either back into the colony or parallel to the agar surface; as a result the lower layer of substrate my-

Figs. 1–5. Actinomyces streptomycini Kras., strains 58 and LS-3; cross-section (2 μ) of 2-3 day colonies.

1. Strain 58; 2 days. 2. Strain LS-3; 3 days. 3. Strain 58; horizontal hyphal growth at border of zone limiting growth below colony (3 days). 4. Strain LS-3; early growth of sporogenous hyphae (I B). spreading hyphae of the second generation (II r). 5. Strain 58; thickened hyphae of growth phase II in substrate mycelium, 3 days. Carnoy fixation. Stained with methyl green and pyronine.

Symbols: HC–suprasubstrate part of colony; C–substrate mycelium; IIM–primary mycelium of substrate part of colony.

Figs. 1, 2, 3–Mag. 400×; Figs. 4, 5–Mag. 2000×.

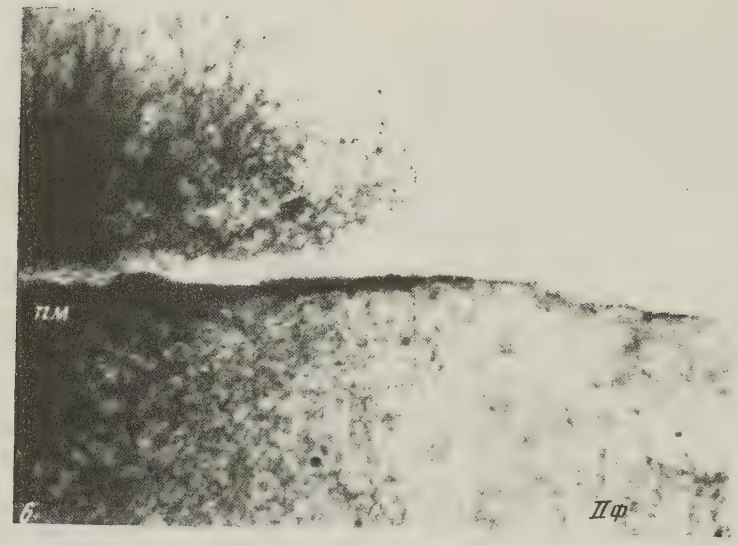
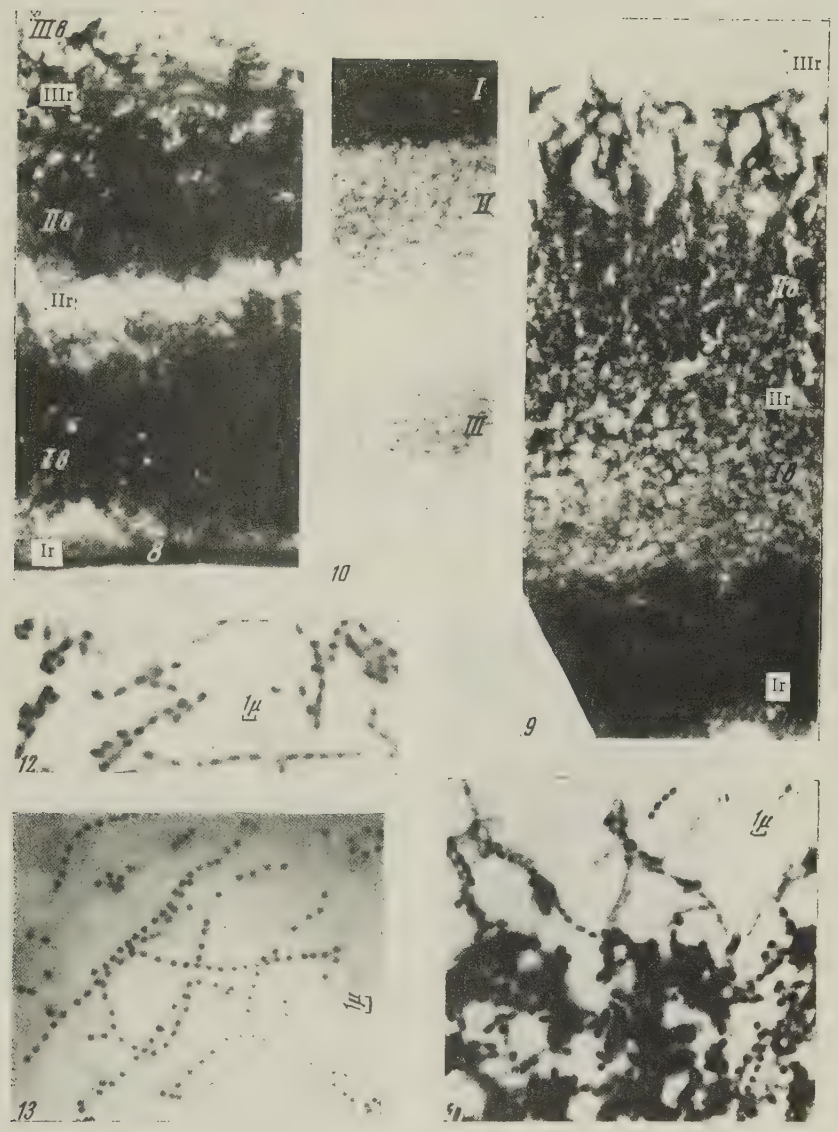


Fig. 6. Cross-section of peripheral part of 3-day LS-3 colony. IIФ—hyphae of growth phase II in substrate mycelium.

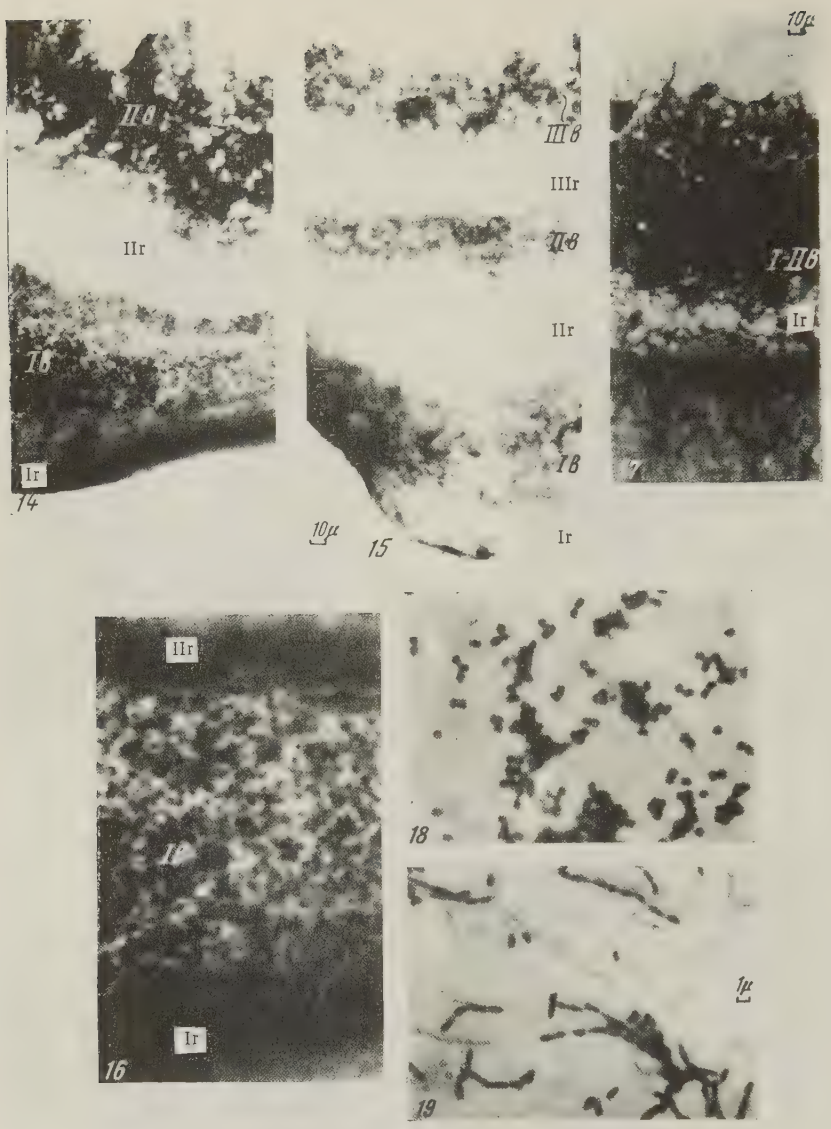
Fig. 7. Strain 58, cross-section of upper part of colony; tuft of spreading hyphae of the third generation (III r) on colony surface, 10 days. Methyl green and pyronine, 2 μ. Fig. 6—Mag. 400×; Fig. 7—Mag. 2000×.



Figs. 8–13. Actinomyces streptomycini Kras., strains 58 and LS-3; 10-day colonies.

8. Strain LS-3. 9. Strain 58; cross-section of colony (2 μ); stratification in structure of suprasubstrate part of colony, due to alternation in growth of spreading (r) and aerial (B) hyphae of three generations of mycelium. 10. Strain 58; substrate part of colony; layering of its structure, due to alternation in growth of hyphae of three generations of mycelium (I, II, III). 11. Strain LS-3; aerial mycelium of the second generation (II B). 12–13. Strain LS-3 sporophores; in offprint. 13–Feulgen stain (nuclear elements); other photographs–methyl green and pyronine.

Figs. 8, 9, 10–Mag. 400×; Figs. 11, 12, 13–Mag. 2000×.



Figs. 14–19. Actinomyces streptomycini Kras., strains 58 and LS-3; cross-section of 25-day colonies. Methyl green and pyronine 14–15. Strain 58, suprasubstrate part of colony. 14–Peripheral part of colony; two generations of mycelium (I and II), vegetative spreading (r) and aerial sporulating (B) hyphae. Hyphae in layer II r have autolyzed. 15–Central part of colony; three generations of mycelium (I r, B; II r, B; III r, B), vegetative spreading hyphal layers I r, II r, and III r have autolyzed. 16. Strain 58. First generation of sporulating mycelium (I B) between layers of vegetative spreading hyphae I r and II r. 17. Strain LS-3. 18. Strain LS-3. Spores. 19. Strain LS-3. Secondary hyphae of substrate mycelium.

Figs. 14, 15, 17–Mag. 400×; Figs. 16, 18, 19–Mag. 2000×.

celium in the 3-day colony is sharply cut off from the medium by the horizontal growth of hyphae (Figs. 2,3) and forms a dome-shaped area within the substrate mycelium. Such a type of growth is apparently related to accumulation in the medium of metabolic products from the culture, unfavorable to growth. Later, however, at about 10 days, this zone of inhibited growth is filled with expanding hyphae.

In the suprasubstrate part of the 3-day colony, a lower layer of horizontally disposed hyphae can be distinguished that is especially strongly developed in strain 58. On the surface of this layer a feltlike mat of mycelium grows out, on whose upper part hyphae of the aerial mycelium grow more or less vertically (Figs. 2,6). Cytoplasm of aerial hyphae is weakly basophilic; nuclear elements are large and stain intensely in the Feulgen reaction, indicating a high DNA content.

Even at this time (3 days), on some hyphae of the aerial mycelium, spore-bearing branches are observed which reach full development by 10 days (Fig. 4). Among the sporophores on the third day there appear solitary vegetative hyphae, growing horizontally and pressing close to the colony (Fig. 4). On growing out, these hyphae later form the lower layer of mycelium of the second generation.

In 10 days the colony growth reaches its full development. Substrate mycelium at this time is formed of three alternating layers of dense and loose growth—I, II, III (Fig. 10). The upper very dense, sharply basophilic layer is formed by mycelium of primary growth in the colony. Autolyzing hyphae are observed in lower layers.

The suprasubstrate part of the 10-day colony is a complex system of layers of three generations of mycelium arranged on top of one another (Figs. 8 and 9: Ir, B; IIr, B; IIIr, B). The lower layer of mycelium adjacent to the agar, as before, is formed by horizontal growth of primary vegetative hyphae (Ir). On this layer the aerial mycelium of generation I (IB) develops, which is a layer entirely of sporulating sporophores. Nonsporulating hyphae are autolyzed; thus, the life cycle of this generation of aerial mycelium ends in full spore formation. However, the presence of nutrients in the substrate and in the mass of autolyzing hyphae stimulates the growth of a new second generation of mycelium.

First the vegetative mycelium grows out, formed by a horizontally growing layer of hyphae (IIr) adjacent to the layer of sporulating hyphae of generation I (Figs. 7, 8, 9), which serves as its substrate. In this layer, there grow out closely-packed vertical sporulating hyphae of aerial mycelium of the second generation (IIB), which are also in a state of full spore formation on the 10th day. On the 10th day, in certain parts of the colony surface, the growth of a third generation of mycelium (Figs. 8, 9) can be observed. Once again, it is formed first of spreading hyphae of vegetative mycelium (IIIr) on which aerial mycelium with sporophores (IIIB) grows out. In strain LS-3, the spreading hyphae of vegetative mycelium in all the generations—Ir, IIr, and IIIr are partially autolyzed, with the result that at their growth sites in the 10-day colony there appear blanks (Fig. 8). At this time in both colony offprints and sections the shape of sporophores and spores, typical of A. streptomycini Kras., is well marked (Krasil'nikov, 1938, 1955). The filaments are straight with chains of closely packed spherical spores, each containing a large nuclear element (Figs. 11, 12, 13). Nuclear elements stain extraordinarily intensely in the Feulgen reaction; methyl green and pyronine stain them a violet color. Thus, in the numerous large nuclear elements of the aerial mycelium are concentrated abundant DNA stores; judging by the violet staining with methyl green, the spore DNA is in a highly polymerized form (Kurnick, 1952). DNA content in the substrate part of the colony is extremely low. A similar observation was made by Italian workers (Giolitti, Craveri, and Corti, 1955). There is reason to assume that the high DNA content in the actively reproducing nuclear elements of hyphae in growth phase II is a most significant factor in spore formation of actinomycetes. A low DNA content in nuclear elements, characteristic of hyphae of the substrate part of the colony, is highly typical of actinomycetes during active growth of the cytoplasmic cell body with inhibition of reproduction of nuclear elements (Prokof'eva-Bel'govskaya, Pestereva, and Rudaya, 1956; Prokof'eva-Bel'govskaya, and Popova, 1959).

The "stratification" typical of colony morphogenesis in A. streptomycini Kras. is due to the shortness of the life cycle in one generation of mycelium, during which it does not consume the nutrients in the colony area. These nutrients are utilized in a number of generations, each of which comprises a regular succession of two types of hyphae: Spreading (vegetative, nutrient hyphae) and vertical (reproductive, sporogenous hyphae). The spreading hyphae participate most actively in the nutrition of the suprasubstrate part of the colony: By taking up with their entire surface the nutrients in the agar medium (hyphae Ir) or the autolysis products from hyphae of mycelium located lower down (hyphae IIr, IIIr), they provide the supply of proteins, RNA, and DNA in the protoplasm that are necessary for the later growth of the aerial mycelium.

After 25 days of growth in both substrate and suprasubstrate parts of the colony, as a result of exhaustion of the medium and accumulation in it of metabolic products, the process of autolysis extends to many areas of the colony. Growth of new generations of mycelium dies out. Colonies undergo gradual degeneration.

In the substrate part of the colony, the longest surviving mycelium is found in the region of primary growth in the colony; on the autolysis products of mycelium in this region there grow out the short, segmented, branched, thickened hyphae of secondary growth, with basophilic protoplasm (Fig. 19). Nuclear elements of these hyphae give a very weak or negative nuclear reaction.

In the suprasubstrate part of the colony, the nonsporulating hyphae of both aerial and "spreading" mycelium of all generations undergo autolysis. As a result there appear blanks in the colony between the layers of dense sporulating mycelium, and all the suprasubstrate part of the colony contracts and becomes more delicate; in offprints and sections the

sporophores readily crumble into free spores (Fig. 18). In strain 58 on the 25th day of growth in the central part of the colony there can be distinctly discerned three layers of hyphae of sporulating mycelium, alternating with three layers of nonsporulating vegetative hyphae (Figs. 15,16). The latter have already autolyzed by this time, with the result that the layers of sporulating mycelium in many areas of the colony (IB, IIB, IIIB) are separated from each other by blanks (Ir, IIr, IIIr). That is why the surface of such a colony "collapses" when touched with a loop. Far from the colony center in cross-section two layers of sporulating mycelium are found, separated by an "empty" space of autolyzed vegetative hyphae (Fig. 14). In strain LS-3 the growth of vegetative spreading hyphae is much weaker and the sporulating layers of 2-3 generations of mycelium in the suprasubstrate part of the colony condense together into one layer (Fig. 17).

The regular succession of spreading, nutrient hyphae and of vertically growing sporogenous hyphae in the suprasubstrate part of the colony indicates the two-phase nature of the growth of the actinomycete culture on agar medium, which has also been observed in submerged growth (Prokof'eva-Bel'govskaya and Orlova, 1956). A most important factor in stimulating the transition of hyphae in phase II to sporogenous ones is apparently the active reduplication of nuclear elements and increased DNA content in them, which are provided by free access of oxygen to hyphae of the aerial mycelium. Thus, the actinomycete colony is formed of hyphae differentiated with respect to type of growth, structure, and function: The spreading (nutrient) hyphae of the substrate mycelium, and the vertically growing and sporogenous hyphae of the suprasubstrate part of the colony. The hyphae in the mat-like mycelium in the suprasubstrate part of the colony are an intermediate type. The nature of this differentiation, as in multicellular organisms, is defined by the position of the cell in the system of other cells, its relation to the source of nutrition and respiration, and the phase of culture development; it is closely related to RNA content in the cytoplasm, reduplication of nuclear elements, and the DNA content in them.

As investigations have shown, all these processes in actinomycetes are under the control of hereditary factors (Prokof'eva-Bel'govskaya, Alikhanyan, Kapitonova and Erokhina, 1958) and the medium (Prokof'eva-Bel'govskaya and Popova, 1959).

A number of studies have appeared in recent years which develop the conclusion originally made by Kleineberger-Nobel (1947), that colony growth in actinomycetes is associated with a succession of two phases: The primary mycelium of the haplophase forms the initial cells from which the secondary mycelium of the diplophase develops, forming spores (Gregory, 1956; Wilking and Rhodes, 1955; Saito and Ikeda, 1959).

The present study did not undertake to investigate this problem. However, we found no indications of growth of the initial cells in the suprasubstrate part of the actively sporulating colonies in strains 58 and LS-3; at the same time we observed in the substrate mycelium the growth of thickened hyphae with distinct nuclear elements (Fig. 5), very similar to the secondary hyphae of the diplophase, described by the authors cited. These factors cause us to favor the conclusion of Erikson (1947, 1948, 1949), according to which the transition of mycelium from the vegetative to the reproductive phase is not due to a sexual process, but to cyclical changes in structure and physiological properties of mycelium and to cultural conditions.

SUMMARY

1. The colony of Actinomyces streptomycini Kras. (strains 58 and LS-3) is formed of hyphae differentiated with respect to type of growth, structure, and function; the nature of this differentiation, as in higher organisms, is defined by the position of the cell in the system of other cells, its relation to the source of nutrition, and the phase of culture development, and is closely related to RNA content in the cytoplasm, reproduction of nuclear elements, and the DNA content in them.

2. Morphogenesis of the suprasubstrate part of the colony in A. streptomycini Kras., strains 58 and LS-3, is characterized by stratification due to the short life cycle of one generation of mycelium that does not consume the nutrients in the colony area. These nutrients are utilized in a number of generations of mycelium, each of which is a regular succession of two types of hyphae, spreading (vegetative, nutrient hyphae) and vertically growing (vegetative and sporogenous).

3. Abundant DNA stores are concentrated in nuclear elements of the aerial mycelium, providing for active reproduction and spore formation.

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PYRUVIC ACID FERMENTATION BY BUTYRIC ACID BACTERIA

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According to present-day conceptions of the chemism of anaerobic processes, pyruvic acid is invariably an intermediate product in the fermentation of six-membered carbohydrates.

It was also found as an intermediate product in butyric acid fermentation (Peldan, 1941; Lemberg, 1956). The subsequent pathway, however, of pyruvic acid conversion, leading to formation of C-4 compounds, cannot yet be considered as settled. There are reports in the literature on the isolation of one enzyme system or another or on the fermentation of assumed intermediate products, but so far there is no experimentally demonstrated scheme of butyric acid fermentation. Most of the studies in this direction, done with enzyme preparations and processes of synthesis of bacterial cell substance are completely excluded (Cohen and Cohen-Bazire, 1948; Nisman and Mager, 1952; Wiesendanger and Nisman, 1954). Under such conditions it is difficult to understand the biological significance of the processes that occur. More fruitful is the method of studying fermentation in living, actively multiplying cultures.

The butyric acid group of bacteria is considered to be very exacting in its nutritional requirements. They are usually cultivated on complex media containing starch, peptone, yeast extract. These natural substrates contain in adequate quantity and suitable form the nitrogen sources and vitamins necessary for growth. It is difficult, however, to study metabolism on such complex media, and hence the desire is understandable of a number of workers to choose synthetic media with an assortment of components of known composition. It has been shown (Wiken, Richard and Aebi, 1952; Wiken and Richard, 1952) that certain strains of butyric acid bacteria can be cultivated on synthetic media containing an assortment of amino acids, biotin, and para-aminobenzoic acid. Good growth of butyric acid bacteria was obtained on inorganic medium nitrogen in the form of $(NH_4)_2PO_4$, with a biotin supplement (Neronova, 1952).

The purpose of the present work was to select the simplest synthetic medium and study pyruvic acid fermentation upon it during growth of butyric acid bacteria, and to compare this fermentation with growth on medium containing pyruvic acid and peptone.

The study was made on a culture of butyric acid bacteria isolated from soil. Fermentation proceeded at 37 deg in tall tubes of 300-350 ml volume or in a special apparatus allowing collection of gaseous products. pH was adjusted by adding the necessary amount of NaOH. Seed material was a 24-hour culture grown from spores on glucose-peptone medium. One per cent of this culture was added to experimental vessels. Samples were taken during the fermentation in which the following determinations were made: pH--electrometrically, cell number--nephelometrically, gaseous products--in a VTI gas analyzer, pyruvic acid--by the bisulfite method (Meshkova and Severin, 1950); volatile acids were distilled off with steam from fermentation fluid in the apparatus of Frolov-Bagreev. It was found that under these conditions pyruvic acid volatilizes along with the fatty acids and later interferes with the determination. After trying a number of methods, the method of quantitative precipitation of pyruvic acid with 2,3-dinitrophenylhydrazine (Houghton, 1934) was chosen. The fermentation fluid was subjected to distillation after being freed of pyruvic acid. The small amounts of volatile acids formed during pyruvic acid fermentation prevented determination of their quantitative and qualitative composition by ordinary methods. Fractional distillation according to Du Claux or Virtanen, ester formation, salt determination, and other methods assume rather large amounts of acids in the solution investigated. Hence we employed the chromatographic method of analysis. The method was based on that described by Kretovich, Drozdova and Petrova (1951). However, we (in collaboration with N. V. Pomortseva, aspirant in the MGU Department of Microbiology) introduced certain modifications in technique in the course of the study. Thus, we used silica gel after one passage through No. 0.1 screen to prepare the column; more concentrated solutions of bromcresol green and sulfuric acid were employed; certain refinements in the technique of column preparation were introduced; a different method of quantitative determination of acids was employed: A solution of acid in chloroform was diluted from alcohol and titrated with NaOH. After modification, the method was found to be very convenient, since in the presence of two volatile acids (acetic and butyric) in the solution it permitted the determination to be made in 20-30 minutes. A detailed description of the method we used is given below.

The silica gel is prepared from one volume of sodium silicate solution and six volumes of water and carefully mixed in a large beaker. To the mixture is added 0.1% thymol blue solution to a light blue color and then concentrated HCl dropwise with continuous careful mixing with a glass rod. Shortly before the color change the solution is converted to a thick gel-like mass. At this point HCl addition should be stopped and careful stirring continued until the gel again becomes liquid. Then HCl is added once more until a pink color appears. In this condition the gel is left overnight. On the following day the layer of clear liquid formed over the gel is poured off by means of a syphon and a fresh portion of water is poured over the gel. The next day the upper layer is again removed and the gel is transferred to a

Buchner funnel, filtered through linen, and thrice washed with water. The washed gel is transferred to a beaker and covered with 1/5 N HCl. Gel ripening lasts 2 days. Then the gel is again filtered through linen on a Buchner funnel and washed repeatedly, till disappearance of an acid reaction (to Congo red). The washed gel is transferred from the Buchner funnel to glass and put in a drier heated to 110 deg. Drying of the silica gel is one of the most important factors in the success of preparation. Hence, it is of much importance to determine correctly when to terminate drying. As the silica gel dries it should be stirred occasionally, breaking up large pieces. Appearance of glass-like particles must not be permitted, indicating that the gel is too dry. Normally dry gel particles can be freely ground in the fingers and there should be no moisture. The dry silica gel can be kept in a vessel with a ground stopper. To prepare a chromatographic column, the silica gel is ground in a porcelain mortar and passed through a No. 0.1 "SATOE" type screen. The powder obtained can be employed for a number of experiments, if it is kept in a hermetically closed vessel. All apparatus—flasks, pipettes, tubing—used in the work should be dry. A glass tube with a drawn-out end is used in setting up the column. Tube length is 25-30 cm, diameter 0.6-0.8 cm. A small piece of cotton is placed in the drawn-out end. 0.5-0.6 g silica gel is mixed in a mortar with one drop of 0.5% bromcresol green solution and one drop of 10% NH_4OH to an even light blue color. To this is added 5-10 ml of a freshly prepared 1% solution of butanol in chloroform and the mass obtained is transferred to the prepared glass tube where it is packed with compressed air delivered from the top through rubber tubing. The column is considered ready at the point when the settling gel mass no longer leaves traces on the tube walls and a clear zone appears on the glass over the packed gel. The finished column is light blue. To the column surface are applied the acids being tested, which have been prepared in the following way: Acids were distilled from the culture fluid with steam. The necessary amount of distillate was neutralized with 0.1 N NaOH in the presence of phenolphthalein in an amount so that acid content of the solution did not exceed 1.5-2.0 ml 0.1 N acid. The salts of the acids obtained were evaporated in a porcelain dish to dryness. For transfer to the column, the acid salts are treated with 2-3 drops of concentrated H_2SO_4 and several crystals of Na_2SO_4 (anhydrous), and the acids liberated from the salts are extracted with 0.5-1.0 ml 1% butanol in chloroform solution. Extraction is repeated 5-6 times, successively transferring with a pipette the solution of acids to the column surface so that at the end of this procedure 5-6 ml of fluid are in the tube. Then the solution of acids is drawn through the column by means of compressed air at a rate of about 120 drops per minute. As the solution flows through, solvent must be added and care taken that air does not get into the column. As the acids move through the column they are separated and form zones of different colors, making it possible to readily establish their qualitative composition. Acids with a large number of carbon atoms move faster, as for example butyric acid. It colors the column green and can be completely eluted in 10 minutes. The shorter the carbon chain the slower the acid moves, and formic acid, coloring the upper part of the column white, does not move at all. Acetic acid appears as a bright yellow zone located under formic acid and is eluted several times more slowly than butyric acid. Propionic acid occupies a position intermediate between acetic and butyric and gives a yellow-green zone.

For quantitative determination each acid was collected in a separate flask. The solution of acid in chloroform obtained was diluted with an equal volume of 96 deg ethyl alcohol and titrated in the presence of phenolphthalein with 0.1 N NaOH. Under these conditions the color transition is seen very well. Prior to experimental determinations a control determination must be run with standard solutions to determine the error of the given batch of silica gel. Properly dried gel gives an error not exceeding 6–8%. If the silica gel is too dry its adsorptive properties, increase and the error may reach 15%. On such gel the acids separate very slowly and it is difficult to elute them.

The first experiments on pyruvic acid fermentation were on medium containing pyruvic acid 0.6%, peptone 1.0%, K_2HPO_4 0.1%. It was found that growth on such medium is possible only at pH below 7.0 (6.0-6.57). Fermentation does not set in at initial pH of 7.1 or higher. Hence, initial pH was adjusted to about 6.5 in all experiments on pyruvic acid. The experimental data shown in Fig. 1, where fermentation products are expressed in millimoles of triose, show that the main fermentation product under these conditions is acetic acid, whose formation begins with culture growth. Butyric acid is formed in considerably smaller quantities and appears only after 12 hours of growth. Pyruvic acid consumption is closely related to increase in

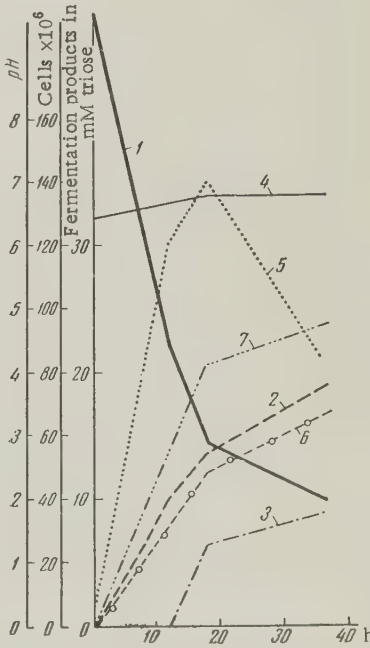

Fig. 1. Fermentation dynamics on medium containing pyruvic acid and peptones. 1) Pyruvic acid; 2) acetic acid; 3) butyric acid; 4) pH; 5) cells; 6) H_2; 7) CO_2.

cell number, and comparison of the amount of pyruvic acid consumed and total acids formed shows that pyruvic acid is evidently under these conditions partially involved in anabolism. Gaseous products consist of carbon dioxide and hydrogen, about twice as much CO_2 being formed as H_2. In the course of fermentation the medium became somewhat alkaline, which is not typical of the group of butyric acid bacteria that during glucose fermentation reduce the pH to 4.0 by the end of the fermentation. The attempt to artificially maintain pH in the acid range with buffer solutions or titration during fermentation was not successful. There was no growth in either case.

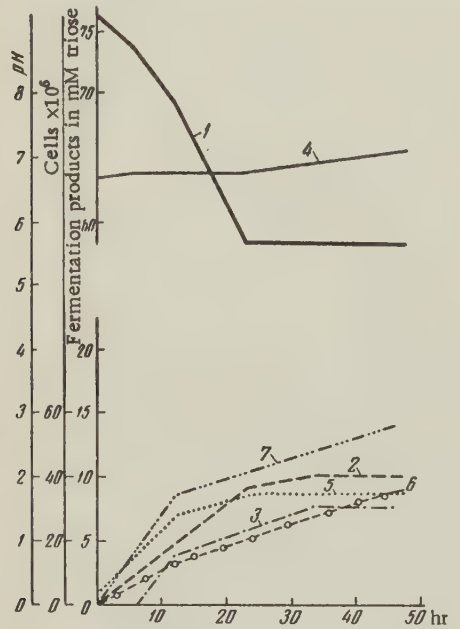

Fig. 2. Fermentation dynamics on medium containing pyruvic acid and aspartic acid. 1) Pyruvic acid; 2) acetic acid; 3) butyric acid; 4) pH; 5) cells; 6) H_2; 7) CO_2.

In connection with data available in the literature, it was of interest to investigate the possibility of growing the strain of butyric acid bacteria we isolated on simple synthetic medium. It was shown that on glucose-containing media, peptone cannot be replaced by ammonium salt, but on medium containing pyruvic acid it is possible to replace peptone by aspartic acid whose amount should not exceed 0.5%. It is necessary to add as a vitamin source 0.01% (by volume) yeast autolyzate. The bacteria do not develop under these conditions. A study of the dynamics of growth was made on medium containing (in %): Pyruvic acid—1, aspartic acid—0.5, K_2HPO_4—0.1, yeast autolyzate—0.01. The results presented in Fig. 2 show that the pyruvic acid is utilized to a very negligible extent and the fermentation ends with a large amount of unfermented material. Bacterial growth is several times poorer than on medium with peptone. The main fermentation product is acetic acid. Twice as much CO_2 is formed as H_2. A comparison of the amount of pyruvic acid consumed and total products formed shows that the pyruvic acid is used almost entirely in energy metabolism. Anabolism under these conditions probably proceeds from the aspartic acid.

It is interesting to note that on medium of the indicated composition, the aspartic acid can be successfully replaced by certain dicarboxylic acids and ammonium salts, the fermentation activity being related to the degree of oxidation of the dibasic acid added. Thus, on medium containing pyruvic acid and succinic acid the growth is rather poor. If the succinic acid is replaced by the more highly oxidized fumaric acid, fermentation proceeds very actively. Initial pH in all these cases must be about 6.5. With an initial pH value of 7.0 there is no growth.

SUMMARY

1. Butyric acid bacteria ferment pyruvic acid both on medium containing peptone and on medium containing aspartic acid.

2. The main fermentation product is acetic acid in both cases; its formation proceeds parallel to culture growth and pyruvic acid consumption. Little butyric acid is formed.

On the medium containing aspartic acid the growth is poor and the amount of pyruvic utilized is slight, but the general character of fermentation is the same as on the medium containing peptone.

Twice as much CO_2 is formed as H_2 in both cases.

Pyruvic acid is utilized almost entirely for energy metabolism on medium containing aspartic acid, while on medium containing peptone it evidently is partially involved in anabolic processes. The aspartic acid can be replaced by succinic or fumaric acid and ammonium salts.

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FERMENTATION OF CALCIUM SALTS OF ACETIC AND BUTYRIC ACIDS BY CLOSTRIDIUM ACETOBUTYLICUM

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In studying the fermentation by Clostridium acetobutylicum of pentose hydrolyzates of corn stalks in a mixture containing flour mash or corn gluten, we observed a considerable increase in the acetone yields (Nakhmanovich and Shcheblykina, 1959). On replacing half the flour with sugar of a neutralized hydrolyzate of corn stalks the acetone yield increased by more than 25% compared to the control flour mash, while butyl alcohol yield scarcely changed.

It was suggested that the acetone increment may result from fermentation of the calcium acetate that is formed on neutralizing the acetic acid in the hydrolyzate with lime.

As Shaposhnikov (1948) has shown, the acetone-butanol fermentation process has two phases: In the first fermentation stage volatile acids are formed, chiefly acetic and butyric, and formic in a small amount; in the second fermentation stage these acids are consumed by the bacteria with formation of the solvents.

This principle is reflected in most of the suggested schemes for the chemism of acetone-butanol fermentation (Logotkin, 1958), although in the different schemes different hypotheses are proposed for the process of formation of the solvents from the volatile acids.

According to data of Ierusalimskii (1942), in most cases the solvents are formed more actively and with higher acetone yield at low pH values than at high values.

Addition of acetic acid during acetone-butanol fermentation increases the acetone yield, but according to Spikeman it does not affect the butanol yield. Addition of butyric acid increases butanol yield. A C. acetobutylicum suspension in the presence of glucose reduces butyric acid to butyl alcohol (Prescott and Dunn, 1952).

Other studies do not support so strict a relation in the conversion of acetic and butyric acids to the respective solvents only.

Thus, Bernhauer and Kurshner (1935) showed that conversion of these acids to the solvents proceeds variously, depending on the activity of the C. acetobutylicum culture. When employing an active culture, up to 97% of the added acetic acid was converted to acetone. A weakened culture converted only about 30% of the acetic acid to acetone and almost all the rest to ethanol. The same thing occurred upon adding butyric acid to the fermenting mash. An active culture converted it almost completely to butanol, and a weakened one converted only 36% and about 15% to ethanol and acetone.

A more thorough study of fermentation of volatile acids, with use of labeled atoms, was made by Wood, Brown and Werkman (1945). The authors added acetic acid with a C^{13}-labeled carbon atom in the carboxyl to a C. acetobutylicum culture fermenting corn mash. It was found, in contrast to the data previously cited, that more than half the heavy carbon (53%) was in the butanol, in about equal amounts in the carbonyl group and in the β-position. Only about 15% of the C^{13} was found in acetone, about 18% in CO_2, 3% in ethanol, and 3% in butyric acid. This indicates a more complex interaction of the added acid with intermediate fermentation products.

Experiments on addition of butyric acid with labeled carbon showed that 84% is converted to butanol, and about 13% of the heavy carbon added was found in the butyric acid in the fermentation fluid.

Stiles, Peterson and Fred (1929), in studying the nature of the acids formed during fermentation of corn mash by C. acetobutylicum, set up fermentation experiments with calcium carbonate. It was found that the calcium carbonate did not completely inhibit formation of the solvents, the yield of which was about 60% of the control (without calcium carbonate). On the basis of this the authors conclude that the calcium salts of volatile acids are fermented in part.

Rabotnova (1957) reached a contrary conclusion, pointing out that neutralized acids cannot be utilized by bacteria in acetone-butanol fermentation.

EXPERIMENTAL

As a result of hydrolyzing pentosans of corn stalks with 1% sulfuric acid, we obtained a 5% pentose solution having a total acidity of 25 ml 0.1 N acid per 10 ml of hydrolyzate, the acetic acid amounting to 9.5 ml 0.1 N per 10 ml. Thus the hydrolyzate represented an approximately decinormal solution of acetic acid.

Prior to fermentation, hydrolyzates were neutralized with lime to an acidity of 0.7-0.8 ml 0.1 N per 10 ml (pH about 6).

On analyzing the neutralizate for volatile acid content, the same 9.5 ml 0.1 N acetic acid per 10 ml of neutralizate was determined, since in the analysis method used (Bekhtereva and Ierusalimskii, 1937) the acetic acid was displaced from its calcium salt by phosphoric acid.

We tested 0.1 N salt solutions for fermentation. It was considered advisable to investigate the fermentation of calcium butyrate also.

METHODS

1.0 N solutions of acetic and butyric acids were prepared and neutralized with equivalent amounts of chemically pure $Ca(OH)_2$. The salts obtained were fermented with glucose or with flour mash by C. acetobutylicum.

The fermentation medium contained per 100 ml 4 g glucose, 10 ml 1.0 N solution of calcium acetate or

Table 1. Fermentation of Calcium Salts of Acetic and Butyric Acids on Media with Glucose or Wheat Flour

(Total content of salts in fermentation medium corresponds to their 0.1 N solution)

Expt. No. and medium	Ca salt added (10 ml 1 N solution per 100 ml medium)	Content of the solvents in fermentation fluid (g/liter)				Residual sugar (%)	Acidity (ml 0.1 N per fermentation fluid)		
		acetone	butanol	ethanol	total		total titratable	volatile (including that from Ca salts)	
								acetic	butyric
Expt. No. 1, glucose and gluten	Control (no salts)	4.45	7.88	1.98	14.31	0.14	4.5	1.88	0.41
	Ca acetate	5.37	7.22	2.19	14.78	0.14	3.9		
		5.51	7.52	1.79	14.82	0.14	4.5	6.81	1.43
	Ca butyrate	4.26	12.79	2.13	19.18	0.16	1.7	6.48	0.91
		4.35	12.32	1.96	18.63	0.15	1.8		
Expt. No. 2, glucose and gluten	Control (no salts)	4.65	7.95	2.00	14.60	0.18	4.5	1.50	0.90
		4.35	7.43	2.18	13.96	0.17	4.4	1.30	0.80
	Ca acetate	5.55	7.35	2.42	15.32	0.19	3.1	6.80	1.20
		5.37	7.58	1.85	14.80	0.16	3.7	5.70	1.30
	Ca butyrate	4.23	10.73	2.27	17.23	0.35	1.4	5.00	1.10
		4.52	11.40	2.27	18.19	0.16	1.1	5.90	1.60
	Acetic acid*	6.85	7.53	2.53	16.91	0.18	5.0	2.20	0.50
Expt. No. 3, wheat flour mash	Control (no salts)	4.55	7.64	1.70	13.89	0.14	4.2	2.26	0.46
	Ca acetate	5.42	7.58	0.65	13.65	0.09	2.9		
		5.47	7.22	1.27	13.95	0.11	3.0	8.77	1.97
	Ca butyrate	4.35	11.27	0.74	16.36	0.10	3.0	7.01	4.48
		4.21	11.57	0.11	15.89	0.11	2.8		

* Added to several containers during fermentation (4 ml 1.0 N solution per 200 ml fermentation medium 5 times).

calcium butyrate, 2.5 g corn gluten, and nutrient salts (in mg): K_2HPO_4—50; KH_2PO_4—50; $MgSO_4$—20; $FeSO_4$—1; $MnSO_4$—1; NaCl—2.

Media from wheat flour were also prepared; such medium contained per 100 ml 4% starch calculated as glucose, and 10 ml 1.0 N solution of the calcium salt being tested. The control in the experiments was the same medium without calcium salts of the volatile acids.

The flour media were cooked and sterilized at 1.5 atm for 1.5 hours, the glucose media at 0.5 atm for 45 minutes.

To each of the experimental vessels containing 200 ml fermentation medium was added 5 ml acetone-butanol bacteria inoculum (except for vessels 2, 4, 5, and 7 in experiment No. 2, where 25 ml of culture was added). Fermentation proceeded at 37 deg.

Fermentation rate was checked gravimetrically by the amount of gases liberated.

The content was determined in the fermented fluid of the solvents (Nakhmanovich and Shcheblykina, 1958), total and volatile acids (Bekhtereva and Ierusalimskii, 1937), and unfermented sugar (according to Bertrand).

In the analytical procedure, as was mentioned above, acetic and butyric acids were displaced by phosphoric acid from the calcium salts of the volatile acids remaining in the fermentation fluid and were determined together with the free volatile acids in the fermentation fluid.

RESULTS

The results of three experiments on fermenting calcium salts of acetic and butyric acids are given in Table 1.

The presence of calcium acetate in the fermentation medium affected only the acetone yield, which increased by 20-24%. Calcium butyrate increased only the butanol yield by 45-60%. It should be noted once again that the molar quantities of these salts in the fermentation media were the same and corresponded to their 0.1 N solutions.

Volatile acid anions, bound by calcium ions and remaining in the fermentation fluid, amounted to 40-60% (in flour mash 80%) of the amount added to the fermentation medium. The fermentation fluid contained chiefly the acetic acid salt, regardless of whether the calcium salt of acetic or of butyric acid was added.

During fermentation the acid anion of the added calcium salt was utilized for butanol formation, and the calcium ion bound the acetic acid formed during fermentation, which has a higher degree of dissociation than butyric acid*.

* The dissociation constants of 0.1 N solutions of acetic and butyric acids are, respectively, $1.75 \cdot 10^{-5}$ and $1.54 \cdot 10^{-5}$ (Handbook of Chemistry, 1952).

When the same molar amount of acetic acid was added during fermentation, the acetone increment was 52% (experiment No. 2). Obviously only 40-50% of the calcium acetate was fermented.

Calcium acetate activated the fermentation. In experiments with glucose in the presence of this salt the fermentation began sooner and proceeded faster (Fig. 1). A similar occurrence is reported in the recently published work of Schoedler (1958).

A balance of the major fermentation products, expressed in milligram-moles per liter, from data on fermentation experiments with calcium salts and glucose, is given in Table 2. Figures in the columns represent the difference between experimental and control fermentation. Certain changes in the amounts of other solvents and acids can be disregarded in the present case, since under the conditions of acetone-butanol fermentation certain fluctuations are always possible in the yields of individual components formed during fermentation of the products; this is particularly likely under the influence of added calcium salts or acetic acid.

On addition of calcium acetate, the acetone increment was 18–18.5 moles per 95–97 moles CH_3COOH, and for 92 moles of acetic acid the acetone increase was 40 moles.

If it is considered that the formation of acetone proceeds through acetone-acetic acid and, consequently, two moles of acetic acid correspond to every mole of acetone, it can be concluded on the basis of the balance data that about 88% of the acetic acid added was used for acetone formation and about 9% remained unfermented.

During fermentation of the calcium salt of acetic acid, about 40% of the anion went to form acetone and 55-60% of the salt remained unfermented. Thus, the bacteria consumed only about half the acid residue of the salt.

During fermentation of calcium butyrate, over 50% and up to 70% of the anion was converted to butanol; not over 10% of the salt remained unfermented.

During fermentation of both these salts, there remained in the fermentation fluid only calcium acetate, where one acetate anion corresponded to each calcium atom. Possibly the basic calcium salt of acetic acid is formed here.

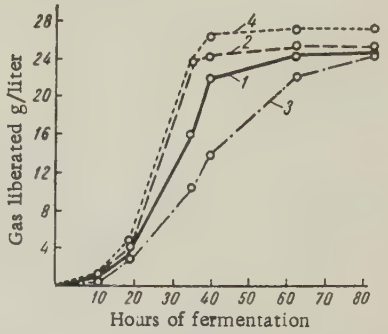

Fig. 1. Fermentation rate according to gas liberation (from experiment No. 2). 1) Medium with glucose and gluten (control); 2) same with calcium acetate; 3) same with calcium butyrate; 4) same with acetic acid.

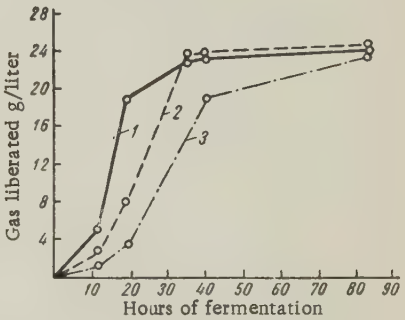

Fig. 2. Fermentation rate according to gas liberation (from experiment No. 3). 1) Wheat flour mash (control); 2) same with calcium acetate; 3) same with calcium butyrate.

Table 2. Balance of Major Fermentation Products (in mg-M/liter)

Calcium salt added calculated as the corresponding acid	Content in fermentation fluid						
	solvents				volatile acids (including that from Ca salts)		
	acetone	butanol	ethanol	total	acetic	butyric	total
None (control)	76.5	106.1	43.0	225.6	18.8	4.1	22.9
Calcium acetate 95.6	95.0 (+ 18.5)	101.3	38.8	235.1	68.1 (+ 59.3)	14.3	82.4
Calcium butyrate 95.0	73.4	172.0 (+ 65.9)	45.7	291.1	64.8 (+ 46.0)	9.1	73.9
None (control)	77.7	104.0	45.4	227.1	14.0	8.5	22.5
Calcium acetate 97.6	95.6 (+ 17.9)	99.6	52.5	247.4	68.0 (+ 54.0)	12.0	80.0
Calcium butyrate 97.0	78.0	154.0 (+ 50.0)	49.3	281.3	59.0 (+ 45.0)	16.0	75.0
Acetic acid 92.2	118.0 (+ 40.3)	101.8	55.0	274.8	22.0 (+ 8.0)	5.0	27.0

SUMMARY

1. The fermentation of calcium salts of acetic and butyric acids by Clostridium acetobutylicum was studied on media containing glucose (4%) and corn gluten or containing flour mash. Concentration of the salts in the fermentation media corresponded to 0.1 N solutions.

It was shown that the salts are fermented in part. Calcium acetate increased the acetone increment by 20-24%, and calcium butyrate increased the butyl alcohol increment by 45-60%.

2. Calcium acetate is only 40-50% fermented compared to an equivalent amount of acetic acid. About half the salt remained in the fermentation fluid.

During fermentation of calcium butyrate, the butyrate anions were almost completely consumed by the bacteria (up to 70% was converted to butanol). The butyrate anion in the calcium salt residue was replaced during fermentation by the acetate anion.

3. Calcium acetate activated acetone-butanol fermentation. Fermentation began sooner and proceeded faster in experiments with glucose in the presence of this salt.

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PECIFICITY IN MICROORGANISMS

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By specificity we mean the ability of microorganisms o produce certain reactions of breakdown and syn-hesis or processes in which these occur. Of particular nterest to us are those reactions having theoretical r practical importance. Therefore, we consider mi-roorganisms to be specific which have the ability to roduce such a reaction. Here, when possible, the nechanism of the reaction should be borne in mind, ince certain reactions or processes may proceed by ifferent pathways.

The reactions or chains of reactions produced by nicroorganisms are undoubtedly associated with the netabolism necessary to the very existence of the mi-robe. In studying microorganisms from the point of iew of specificity, attention should be given not only o the biochemical processes occurring in the microbe nd its metabolism, but to morphological and cytologi-al structure as well.

Treatment of the question of specificity in microor-anisms is associated with the names of Pasteur, inogradskii, Bejerinck, and Delbruck. The roots of his idea must be sought in the observations of the mpiricists and technologists among our forefathers. ouis Pasteur, in his study of microorganism activity, as interested in them chiefly as the agents causing ermentation or infectious diseases. Vinogradskii, orking with specific microorganisms, developed a nethod for isolating them on selective media. Bejer-nck (1901) pointed out that in obtaining microorgan-sms we are imitating what happens in nature; Delbruck otes particularly, that in obtaining microorganisms n selective media we are dealing with natural pure ultures.

In the studies of Delbruck, special mention is made f the role of the environment and the struggle for xistence among microorganisms having different netabolic features. The basic principles of Delbruck's heory can be found in the article by F. Hayduck and . Haehn (1931).

We include among the specific microorganisms those hat play a part in industry, agriculture, and natural rocesses not yet utilized by man. Considered to be pecific are nitrifying microbes, microbes that assim-late atmospheric carbon dioxide and carbonates, nicrobes that assimilate atmospheric nitrogen, mi-robes that decompose pectins, the purple bacteria, nd others; among the specific microorganisms are lso included the microbial agents of various infectious iseases.

Pasteur and Vinogradskii believed that certain spec-fic reactions were produced by a restricted group of nicroorganisms. Recent studies show that specificity s now acquiring a different aspect, that the range of nicroorganisms having a certain specificity is not as estricted as it appeared earlier.

In analyzing the question of specificity in the present paper, of particular significance to us was the discovery of the necessity of CO_2 for growth and development of heterotrophic microorganisms and the studies on the nature of the microorganisms participating in petroleum decomposition and methane formation. Let us proceed to a discussion of the experimental data giving evidence regarding this.

Microorganisms that Produce Destruction of Pectins.

In 1895, S.N. Vinogradskii made a report to the Academy of Sciences in Paris of work on the isolation of a microorganism that decomposes pectin during retting of flax; he concluded the report with the following words: "We have here a new and curious example of extreme specialization of function in the world of microbes." This microbe was subsequently named Plectridium pectinovorum; it belongs to the group of microbes that ferment butyric acid.

Later, in various laboratories at home and abroad, a number of bacteria and molds were isolated that decompose pectin during maceration of various plants. These microorganisms, adapted to various ecological conditions, differ from one another in the cardinal points of pH, temperature, tolerance to atmospheric oxygen, etc. (Seliber, 1951, 1953; Raynaud, 1949).

Among anaerobic pectin-decomposing microorganisms, species were found able to reduce sulfate (Alekseev, 1951; Prevot, 1948).

Among aerobic pectin-decomposing bacteria, the presence was shown of Bacillus macerans, B. subtilis, B. mesentericus, and certain forms of Pseudomonas genus.

A part is played in the microbiological processes occurring during the spreading of flax not only by fungi (Cladosporium herbarum and others) but also by yeastlike microorganisms, as well as bacteria. According to K. Wieringa (1956), the fungus C. herbarum predominates in spread flax during summer months, and the rest of the time the yeastlike organisms, Rhodotorula glutinis and Rhodotorula macerans predominate; in spring and autumn, the fungus Pullularia pullulans takes a certain part in the process of pectin decomposition. Bacteria of the Pseudomonas fluorescens type may play an active part throughout the whole year. The pectin-decomposing microorganisms play a large part in decomposition of plant residue in soil. No less considerable is their role in plant diseases, inasmuch as the presence of protopectinase is considered to have been demonstrated in a large number of bacterial plant pathogens.

Microorganisms that Assimilate Carbon Dioxide and Atmospheric Nitrogen.

The phenomenon of autotrophism (chemosynthesis) was discovered by Vinogradskii in the nineties of the

past century; the assimilation of carbon dioxide in nitrifying bacteria and in iron and sulfur bacteria was assumed to occur in this way. But in this century, new data have appeared which showed that almost all, or very many, heterotrophs require carbon dioxide for growth; it has been shown that some of the heterotrophs assimilate carbon dioxide. Thus, the ability to assimilate carbon dioxide cannot be attributed to only a restricted range of microbes. It should be further added that certain protozoa and certain animal and plant tissues are able to fix carbon dioxide.

Another very important element present in the atmosphere and comprising an important constitutent in every organism is nitrogen; this element is also fixed by bacteria.

In 1893, Vinogradskii isolated in St. Petersburg the anaerobic bacterium, Clostridium pasteurianum, that fixes atmospheric nitrogen. The aerobic nitrogen-fixer, Azotobacter, was isolated by Bejerinck in 1901. The ability to fix nitrogen is also present in anaerobic pectin-decomposing bacteria (Bychkovskaya, 1940; Alekseev, 1952).

Bredeman has reported on the nitrogen-fixing ability of B. asterosporus. This ability has been found in the photosynthesizing bacterium, Rhodospirillum rubrum. Ability to fix nitrogen vigorously was found in certain sulfate-reducing bacteria (de Gall, Senez and Pichinoty, 1959). Omelyanskii (1953), giving in his monograph a survey of the literature on nitrogen-fixing microorganisms, reached the following conclusion: "The chief role in binding nitrogen in a layer of soil is played by the bacteria Clostridium pasteurianum, Azotobacter, and B. asterosporus. But, together with these, many other aerobic and anaerobic species participate in this process and, although not fixing nitrogen so vigorously, play a prominent part in the process of nitrogen fixation as a result of their extraordinary prevalance in soil. Many of these microorganisms belong to commonplace species that grow luxuriantly on ordinary broth media." The finding should be noted in connection with nitrogen-fixers among the epiphytic microorganisms—B. herbicola (Novikova, 1952).

Sulfate-Reduction by Microorganisms.

Of greatest interest in this group is Vibrio desulfuricans, isolated by Bejerinck from canal water in Holland. It should be noted that this microbe is able to utilize carbon dioxide autotrophically.

Baumann (1950) and Denk (1950) showed that sulfate-reducing bacteria have the ability to simultaneously reduce nitrates, converting NO_3 to NH_3. Sulfate-reducing bacteria are concerned in the transformation of petroleums and apparently in their formation and the formation of methane.

Among studies dealing with this problem, the work of Shturm (1948) should be mentioned.

Microbes that Decompose Petroleum and Microbes Participating in Its Formation.

Many workers report data on the presence of various microorganisms in the waters accompanying petroleum and in the petroleum itself.

These studies are covered in sufficient detail in the monograph of Beerstecher (1957). We shall not dwell on these studies here. We will touch upon the recent studies of Simakova et al. (1958) in this field. The authors, studying the type of change in petroleums under anaerobic conditions produced by microbes, found that these changes do not occur under the influence of any single group of microorganisms, but as a result of the joint effect of various physiological groups, including sulfate-reducing bacteria and other microorganisms, in particular, Pseudomonas fluorescens denitrificans and other species of Pseudomonas genus. A long list of microorganisms that assimilate hydrocarbons, including various petroleums, is given by Beerstecher (1957).

Regarding microorganisms participating in petroleum formation, Beerstecher writes: "The problem is not yet settled, although some progress has been made toward its settlement. The role of bacteria in this process is considered to be established by many scientists, although it has not yet been sufficiently defined." Studies have been made in this direction with sulfate-reducing and other bacteria by Jankowsky and ZoBell (1944), Prevot (1951), and Seliber (1948).

Bacteria that Form Methane

In connection with the question of the microorganisms that participate in the formation of hydrocarbons and petroleum, the work of the American scientist, Thayer (1931), is of definite interest. This investigator, working with salts of fatty acids and with mud specimens or with bacterial cultures isolated with the purpose of obtaining hydrocarbons with a greater number of carbon atoms, obtained methane. The author gives no description of the culture with which he worked, but says that growth of the enriched culture warrants the assumption that the methane formation was due to sulfate-reducing bacteria. In this author's experiments, 60-70% of the gas formed was methane.

Regarding the question of methane-forming microorganisms, we would point out that Omelyanskii (1953), in his first study on methane, says nothing about his own search for the agent causing this fermentation. Only at the end of a short article on the methane fermentation of ethyl alcohol does he report the following data on this question: "In the initial stage of the studies conducted up to the present, we limited ourselves to experiments on enriching a culture with specific microorganisms and to study of these microorganisms. The agent causing the fermentation is a very thin and rather long nonsporogenous rod, slightly curved. This species grew in the culture in such overwhelming numbers that there was no doubt whatever that it was the agent causing methane fermentation of ethyl alcohol." In his fundamental work on methane, Omelyanskii points out that there are data in the literature on methane liberation during milk sugar decomposition by pure cultures (Baginsky, 1888), and during growth on elastin as the protein (Luigi, 1897).

Unfortunately, we have not the opportunity here to discuss in detail the specific bacteria that form methane or the mechanism of methane formation. We will only mention some data in the literature. Beerstecher says that in seems possible to state that methane can be obtained from various compounds by the action of various microorganisms. In a review article, Genz-

burg-Karagicheva (1958) gives a picture of the agent causing pectin fermentation of proteins, without citing any other data regarding this microbe; the latter differs greatly in external appearance from the specific forms discovered by Barker (1936, 1949); Barker mentions incidentally, in an article of review type dealing with gas formation, that methane is formed from a diversity of organic compounds.

We have demonstrated in a series of examples that the ability to produce certain reactions or chains of reactions, that was earlier attributed to a restricted range of microorganisms, is characteristic of a wide range of microorganisms belonging to various classifications. Of special interest in this respect are the microbes that decompose and, according to some suggestions, form petroleum. As we have indicated, Beerstecher cites a long list of microorganisms (bacteria, fungi, yeastlike microbes, actinomycetes) that decompose hydrocarbons (including petroleum and paraffin). Among these microbes are encountered commonplace forms growing on meat-peptone media. We should add that we could support our conclusions regarding specificity by still further examples. With regard to the question of the decomposition of various petroleums, a more detailed investigation should be made as to what microbes decompose certain petroleums and what microbes decompose others; the same thing is true concerning hydrocarbons in general.

The ability to produce a certain reaction in members of the same species may undergo various quantitative changes (Vorob'eva, 1958). In connection with the question of microorganism specificity, we will give briefly the results of one study made in collaboration with Aristovskaya in 1940-1941.

Since in making determinations on microorganisms we very often restrict ourselves to indicating the formation of acids or bases, we undertook to determine whether a more definite answer to the question of the action of a number of microorganisms on an aqueous sugar solution could not be obtained. We suggested that microorganism action in this case might proceed according to a Cannizzaro type of reaction; in order to learn what might happen here, let us take two links in the chain of the glucose formula

$$\begin{array}{lll} CH_2O & H_2 & CH_3OH \\ + & \longrightarrow & + \\ CH_2O & O & HCOOH \end{array}$$

Thus, from the action of water on one glucose molecule, $3CH_3OH+3HCOOH$ may be obtained.

In his article on methane fermentation of ethyl alcohol, Omelyanskii (1953) writes that molds of Penicillium and Aspergillus genera grow well on 3% methyl alcohol solution. It is also known that certain plants grow in solutions containing methyl alcohol. Our problem, therefore, consisted in testing a number of microbes for consumption of methyl alcohol and formic acid; this we did. We employed as nutrient medium an inorganic solution containing NH_4HPO_4 as nitrogen source, and methyl alcohol or formic acid salt as sole carbon source. It was found that Mycobacterium rubrum, Mycobacterium aurantiacum, B. pyocyaneum, B. galleriae, B. fluorescens, B. prodigiosum, A. niger, A. oryzae, and Actinomyces longisporus ruber 184 grew in the presence of methyl alcohol. Nearly all these microorganisms grew on the medium containing formic acid salt.

The same microorganisms grow on medium containing CH_3OH and HCOOH that are able to utilize each of these compounds separately. Thus it was found that a number of microbes belonging to various classifications have the capacity to assimilate compounds such as methyl alcohol and formic acid; there is also reason to assume that these compounds are intermediate products in glucose decomposition by these microbes.

Before formulating the conclusions from the present study, let us mention the tasks confronting us in the further study of the question that interests us here. First of all, it must be established whether microorganisms producing one specific reaction can produce another specific reaction; then, the metabolism of the specific microbes should be studied, and their enzyme systems. The data obtained should be employed to classify them.

In reviewing the material cited in the present article, we may say that many specific microbes, of both theoretical and practical interest, have been discovered in recent decades; it has been found, in connection with the demonstrated importance of carbon dioxide for activity of heterotrophs, that many members of this microbial group are able to assimilate carbon dioxide; it has been found that certain purple bacteria can fix atmospheric nitrogen; the ability to fix atmospheric nitrogen in small amounts was found in a number of oligonitrophils.

In medical microbiology many new agents causing virus and bacterial diseases have also been discovered in recent decades.

It is evident from what has been said above, that in the past half-century a number of new microorganisms have been discovered and new specific properties have been discovered in a number of already known microbes; therefore, it should be assumed that there still exist in nature a considerable number of unkown microbes and that microbes already known may possess new properties as yet undetected. Consequently, the point of view from which the question of specificity is regarded in the present article is of heuristic significance. This point of view tells the investigator that, in view of the data on specific microbial properties cited in the present article, one must always bear in mind that in addition to now known agents causing specific reactions, there may exist agents which are as yet unknown.

SUMMARY

1. Carriers of specificity (ability to produce specific reactions) in a number of theoretically and practically important microbiological process may be found in a wide range of microorganisms rather than a restricted one.

2. The same microorganism may produce different specific reactions; the same specific reaction may be produced by different microorganisms.

3. In studying microorganisms, more attention should be given to metabolism.

4. In classifying microorganisms, cytological structure and physiological properties, and in particular, reaction capacity, should be considered as well as morphological properties.

5. The rules established in the present paper for saprophytes should be adopted with certain restrictions by medical microbiology.

6. In searching for agents causing a specific reaction, one should not stop at what has been accomplished. The possibility of the presence of as yet unknown agents in the reaction must be borne in mind.

7. A more conscious application of the methods of ecological microbiology to various fields of applied microbiology, including the medical field, should lead to more profound understanding of the role of microbiological processes in our natural surroundings and elucidate the role of individual microbes or groups of microbes in these processes.

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STUDY OF ANAEROBIC PROACTINOMYCETES
I. ISOLATION OF PURE CULTURES FROM NATURE

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Despite an enormous literature on the investigation of ray fungi, the biology and classification of various groups are obscure and information often contradictory. This is particularly true of the so-called anaerobic proactinomycetes.

We are employing the term proactinomycete here in the broad sense and somewhat arbitrarily, chiefly in connection with the fact that in Krasil'nikov's Determinative Manual (1949), the designation Proactinomyces israeli was adopted for one of the typical anaerobic representatives of the ray fungi.

As is known, Waksman (Waksman and Henrici, 1943) and also the editors of the latest edition of Bergey's Determinative Manual (1957) deem it necessary to put such organisms in a special Actinomyces genus containing 2-3 species.

This treatment has encountered a number of objecttions regarding both designation and size of the group as a whole (Rosbury, 1944; Prevot, 1948) and its position among other classified groups (Haupt and Zecki, 1933). It has been proposed (Frank and Skinner, 1953) to expand this group, including in it the so-called anaerobic diphtheroids (King and Meyer, 1957). Extremely few works have dealt with comparative study of the anaerobic proactinomycetes and morphologically and biologically similar organisms. Data on their prevalence in nature are very limited. Following the work of Wolf and Israel (1891) and Naeslund (1931), it was generally assumed that organisms of this group are not encountered in natural surroundings but inhabit the oral cavity of man and animals, where they are usually sought (Bibby and Knighton, 1941). Recent reports, however, indicate that the anaerobic proactinomycetes are more widely distributed (Hvid-Hansen, 1951; Beerens and Hvid-Hansen, 1952).

The purpose of the present study was to investigate the prevalence of anaerobic proactinomycetes in certain natural sources on the one hand, and to obtain pure cultures for further systematic study on the other.

No clinical material was studied in this work. In isolating new strains from the environment, we employed for comparison cultures of A. israeli, A. meyeri, and A. abscessum. These organisms can apparently be regarded as typical members of the anaerobic proactinomycete group. They were kindly put at our disposal by Professor Prevot from the collection of the Pasteur Institute in Paris.

The basic characteristics, which we regarded as sufficient to classify an organism, isolated in pure culture, in the anaerobic proactinomycete group, were the following: Cells are rod-shaped, size not uniform, shape sometimes irregular. Often distributed at an angle. V- and Y-forms are encountered. Branching was definitely noted in some strains, in others was more difficult to determine. Nonmotile. Gram-positive. Non-acid fast. Conventional anaerobes (do not grow in the presence of air, but retain viability for more than a week). Form glossy white colonies 1-4 mm in diameter of paste-like consistency under anaerobic conditions on protein media (meat-peptone agar with glucose, peptone-corn agar). Even colony border. In semiliquid medium (containing 0.15—.20% agar) growth along the stab one cm from the surface. In liquid medium growth is in the form of a cottony deposit on the bottom, medium is slightly turbid (more or less according to strain). Do not grow on media containing inorganic nitrogen source. Do not grow below 30 deg. Growth relatively slow, becomes noticeable at the end of the second or on the third day. Carbon sources are fermented with formation of acids; gas is never formed.

METHODS

For isolating anaerobic proactinomycetes from the environment, we seeded the respective inoculum (soil, water, etc.) on agar medium in Petri dishes or in the depths of a column of hardening agar in tubes. The inoculated plates were put in a microanaerostat of the MI type from the Leningrad electromedical equipment plant. Air was removed from the anaerostat with the aid of a vacuum oil pump to a residual pressure of 10 mm and was replaced with commercial nitrogen. The procedure was twice repeated. The 0.5-0.7% O_2 present in the commercial nitrogen was absorbed with a moist mixture of pyrogallol A and soda, which was placed in the anaerostat immediately prior to removing air. Since a saturated soda solution was present here, the partial pressure of CO_2 over it was constant, amounting to 0.14%. Attainment of anaerobic conditions was checked with the aid of the indicator of Ulrich and Larsen (1948).

When tubes containing hardening agar were inoculated, in order to produce anaerobic conditions, the top of the column with the inoculum was covered with a two-centimeter layer of aqueous agar containing 1 g/liter sodium hydrosulfite.

Incubation was conducted at 37 deg for 6-8 days. At the end of this time the plates were removed from the anaerostat, tubes cut apart, and colonies seeded on agar medium in plates. Some of the latter were incubated under anaerobic conditions, some under aerobic conditions (as control for growth under aerobic conditions). As a result of seedings with repeated microscopic examination, a pure culture was obtained. Transfers and maintenance and storage of cultures were conducted in semiliquid medium (containing 0.15-0.2% agar) poured as a tall layer in tubes. Prior to inoculation such medium was heated 10 minutes in boiling water and then cooled rapidly. Cultures were stored in the refrigerator at 3 deg.

The basic medium in all experiments was meat-peptone agar containing 0.5% glucose and 0.05 g/liter sodium hydrosulfite. In the experiments on isolating anaerobic proactinomycetes from mineral hydrogen-sulfide waters, we also employed medium SR-2 of the following composition (in g): $(NH_4)_2SO_4$—3; $MgSO_4 \cdot 7H_2O$—1: $CaSO_4 \cdot 2H_2O$—5; K_2HPO_4—0.5; NaCl—10; $CaCO_3$—1; calcium lactate—10; glucose—5; peptone—2.5 (or 10% yeast autolyzate—25 ml); iron lactate—0.5; agar—15; tap water—1 liter; pH 7.0-7.2. This medium was used in some experiments because anaerobic proactinomycetes and certain sulfate-reducing bacteria could grow upon it simultaneously.

In analyzing air for content of anaerobic proactinomycetes, we employed either of two means: 1) Open Petri dishes with meat-peptone agar containing glucose and hydrosulfite were held in the air for a definite time interval, then covered and put in the anaerostat; 2) an open plate with medium was put in a closed vessel, an inlet tube 1 cm in diameter and 1.5 cm in length ended 2 mm from the medium surface in the plate. Air was passed through the vessel with the aid of a water-jet pump at a rate up to 20 liters per minute. Measurement of the volume of air passed through was made with a gas counter where the air entered from the vessel with the plate.

DISCUSSION OF RESULTS

The materials from which attempts were made to isolate anaerobic proactinomycetes were mineral hydrogen-sulfide waters, soil, and air.

A total of 97 specimens of natural waters were analyzed, containing various H_2S concentrations or no H_2S. Analysis was made of deep ground waters, deep waters appearing in well drillings, and hydrogen-sulfide waters accompanying petroleum deposits. Each specimen was seeded on 10 plates with meat-peptone medium, 0.1 ml on each.

Table 1 contains a list of the water specimens in which anaerobic proactinomycetes were isolated. The hydrogen-sulfide mineral waters appearing in well drillings in the region of Pyatigorsk were analyzed in greater detail. Each type here was analyzed three times (each time with a fresh sample of water).

Table 2 contains a list of the hydrogen-sulfide sources in Pyatigorsk where anaerobic proactinomycetes were isolated. In the experiments whose results are shown in Tables 1 and 2, however, not less than 4-6 days elapsed between sample collection and analysis. Therefore, a number of experiments were done on isolation of anaerobic proactinomycetes from hydrogen-sulfide waters in the region of Pyatigorsk immediately at the site, where inoculations were made not later than 3-4 hours after sample collection. In these experiments 3-5 ml of the water specimens were seeded in tubes containing hardening meat-peptone agar or SR-2. After incubation, tubes were cut apart and colonies seeded in Petri dishes containing meat-peptone medium. Plates were incubated under anaerobic conditions. Thus more than 130 colonies were seeded and checked; however, growth of anaerobic proactinomycetes was obtained in only 10 cases (Table 3). On seeding the colonies grown in a column, solitary colonies of anaerobic proactinomycetes usually developed on the plate. Massive growth of these colonies was observed in only two cases (specimens of water from drill hole No. 16 and "Warm Narzan" No. 7).

A similar situation was observed in all cases of seeding water specimens directly in plates. In the great majority of cases there were no colonies of anae-

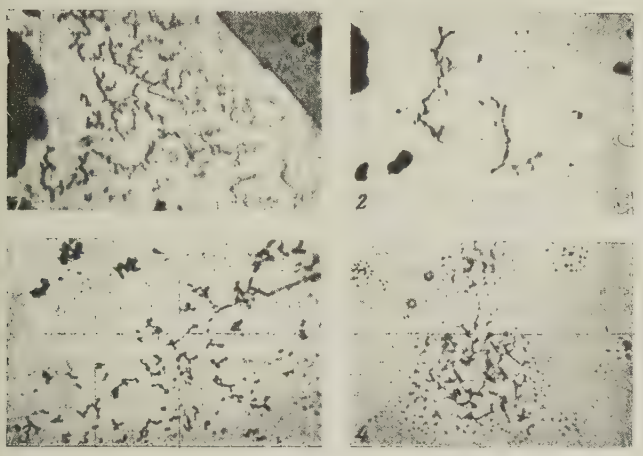
Table 1. Water Specimens in Which Anaerobic Proactinomycetes were Isolated

Site of sample collection	Source	Brief hydrochemical description of water			No. of strains isolated
		type of mineralization	total H_2S, g/liter	temperature	
Krasnodar krai, Goryachii Klyuch health resort	Drill hole No. 32	HCO_3^-—Cl'—Na^+	0.0026	47°	5
	Drill hole No. 37 (repeated)	HCO_3—Cl—Na^+	0.1098	46.5°	3
	Drill hole No. 63 (repeated)	Cl'—SO_4^{2-}—Na^+	0.1453	42°	1
Groznyi oblast, Sernovodsk health resort	Drill hole No. 7	HCO_3^-—SO_4^{2-}—Na^+	+	19°	1
Tbilisi	Drill hole No. 2		0.0193	42.4°	3
Baku	Ground waters of petroleum deposits	—	+	—	7
Baku (2nd)	Ground waters of petroleum deposits	—	+	—	2
Nal'chik	River Belaya weakly mineralized hydrogen-sulfide waters				1
Black Sea	Latitude N. 44° 33'. Longitude E. 40° 41'	Depth—500 m	H_2S zone	—	2
Moscow	Water from metro tunnel	—	—	—	18

Note. Data on hydrochemical description of waters are taken from those of health-resort observation stations. The sign (—) signifies that we have no data at our disposal; the sign + signifies the presence of H_2S determined qualitatively.

robic proactinomycetes in the plates. Where they did grow the number was low; usually 1-3, 4 was the exception. The percentage in this case of anaerobic proactinomycete colonies, of the total number of microorganism colonies growing on the plates, varied within a wide range, 2-5 to 20-25.

On analyzing the data in Tables 1, 2, and 3, no relation between the prevalence of anaerobic proactinomycetes in various waters and the hydrogen sulfide content or temperature of the water could be found. It is also evident from Table 3, that use of SR-2 medium in isolating anaerobic proactinomycetes has no appreciable advantage over meat-peptone medium.



Figs. 1–4. Microorganisms growing on slides left for 8 days in stream of water source Lermontovskii No. 1 Pyatigorsk. Stained with erythrosin. Mag. 900×.

The growth of solitary colonies of anaerobic proactinomycetes on Petri dishes always raises doubt regarding the source whence this organism got into the plate. In order to eliminate this doubt, we employed the method of growth slides to analyze certain waters. Sterile glass slides were placed in the stream in the drill hole pipe of the water source Lermontovskii No. 1 in Pyatigorsk for a period of 8 days. Then the slides were removed, fixed with heat, and stained with 3% carbolic erythrosin. Relatively abundant growth of microorganisms could be observed on the slides (Figs. 1, 2, 3, and 4), which, judging from morphological characteristics, can be classified with the proactinomycetes or mycobacteria.

Growth slides in another experiment from the same source were not fixed, and imprints were made from these slides to the surface of meat-peptone agar in Petri dishes. Plates were incubated under anaerobic conditions. Here, it was possible to observe growth of anaerobic proactinomycete colonies. The colonies grew only in the section of the medium to which the growth slide had been applied when imprints were made. A number of these colonies reached 4-5 per plate, comprising up to 40-50% of the total number of microorganism colonies growing under these conditions.

Table 2. Isolation of Anaerobic Proactinomycetes from Hydrogen-sulfide Water Sources in Region of Pyatigorsk

Source	Hydrochemical description of water			No. of strains isolated
	type of mineralization	total H_2S, g/liter	temperature	
Narodnyi	$Cl'-HCO_3'-Na'-Ca''$	0.0098	46.9°	11
Lermontovskii No. 2	$Cl'-HCO_3'-Na'-Mg''$	0.0104	35.4°	5
Inclined drill hole	$Cl'-HCO_3'-Na'-Ca''$	0.0107	47.4°	4

Table 3. Isolation of Anaerobic Proactinomycetes from Hydrogen-Sulfide Water Sources in Region of Pyatigorsk (Inoculation not later than 3-4 hours after sample collection)

Source	Hydrochemical description of water			Total strains isolated	Medium	
	type of mineralization	total H_2S, g/liter	temperature		MPA with glucose	SR-2
Drill hole No. 1, Essentuki	$HCO_3''-Cl'-Na'-Ca''$	0.0200	23°	4	1	3
Drill hole No. 16, Pyatigorsk	$Cl'-HCO_3'-SO_4''-Na'-Ca''$	0.0100	48°	1	0	1
Inclined drill hole, Pyatigorsk	$Cl'-HCO_3'-Na'-Ca''$	0.0107	47.4°	2	1	1
Lermontovskii No. 1, Pyatigorsk	$Cl'-HCO_3'-SO_4''-Na'-Ca''$	0.0097	47°	2	1	1
"Warm Narzan" No. 7, Pyatigorsk	$Cl'-HCO_3'-Na'-Ca''$	Traces	35.4°	2	2	0

Table 4. Isolation of Anaerobic Proactinomycetes from Air of Laboratory Premises

Volume of air passed through, liters	Average No. of microorganism colonies per plate	No. of anaerobic proactinomycete colonies per plate
5	4	0.3
20	5.6	0.31
100	9.2	0.4
200	11.8	0.48

Note. These data are averages of three experiments. In each experiment the figures for each variant are the average of 10 replicates. Medium—MPA with glucose and hydrosulfite.

Table 5. Isolation of Anaerobic Proactinomycetes from Air of Premises in Relation to the Presence of People

Exposure site of open Petri dishes	Exposure time (hours)	Average No. of microorganism colonies per plate	No. of anaerobic proactinomycete colonies per plate
Premises where people are constantly present	1.5	71	9.0
Well-ventilated premises where people are not constantly present	10	18	0.13
Pine park	10	5	0.0

Note. These are average data from three experiments. In each experiment the figures for each variant are the average of 10 replicates.

These experiments demonstrate that anaerobic proactinomycetes actually occur in certain natural waters and can live there in greater or lesser numbers. We isolated a total of over 80 strains of these organisms from various waters.

Isolation of Anaerobic Proactinomycetes from Air.

In the literature known to us, there are no data on finding anaerobic proactinomycetes in air. Various contradictory references concern Mycobacterium (Corynebacterium) acnes (Lentze, 1950; Gyllenberg, 1958)--an organism similar to the anaerobic proactinomycetes but differing from them in colony color and certain other properties. A number of experiments were done in order to determine how prevalent anaerobic proactinomycetes are in the air of buildings.

Average data from three such experiments are given in Table 4.

As is seen, anaerobic proactinomycetes are relatively prevalent in the air of laboratory premises. In order to determine to what extent their presence in the air is related to the presence of people on the premises, a number of experiments were done outside the city of Moscow, 20 km east of it. In these experiments the presence was determined of anaerobic proactinomycetes in the air of premises where people spend a varying amount of time and, for a control, in the air of a pine-forest park. The average data from three such experiments are given in Table 5.

It can be concluded from these data, that the presence of anaerobic proactinomycetes in the air of buildings is related to the presence of people in them. Apparently, the members of this group of organisms encountered in the air, are those associated with the normal human microflora.

We isolated from air in pure culture, a total of over 70 strains of anaerobic proactinomycetes.

Attempts to Isolate Anaerobic Proactinomycetes from Soil.

These attemps invariably ended in failure, despite the fact that a considerable number of soil specimens of different types were analyzed. The negative results from these experiments can be explained either by an absence of anaerobic proactinomycetes in the soil, or by the fact that their number is not high, and in the absence of selective media we could not detect them.

In conclusion, the author expresses his deep gratitude to Professor N. A. Krasil'nikov, under whose direction this work was carried out.

SUMMARY

A study was made of the prevalence of the anaerobic proactinomycete group in various natural sources. Cultures of these organisms were isolated from natural waters and air of premises where people are present. Attempts to isolate anaerobic proactinomycetes from soil failed.

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THE TRANSFORMATION OF FATS THROUGH THE ACTION OF MICROORGANISMS
1. THE DECOMPOSITION OF FATS DURING THE COURSE OF DENITRIFICATION

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Various organic residues of animal, plant, and mixed origin have been proposed as the starting material for the formation of petroleum. Many modern theories have assumed that lipoids (fats, fatty acids, waxes, sterols, resins, plant pigments) are the initial parent substances of petroleums. The close structural analogy between the hydrocarbons of the paraffin series and fatty acids makes it possible to consider fatty substances as the most probable precursors of petroleum hydrocarbons. A number of experiments confirmed the probability of this sort of hypothesis. Engler, Kobayashi and Yamamoto, Stadler and Kunkler (1937), and Zelinskii (1941) experimentally obtained petroleum-like products from fats of various origins by chemical means; moreover, depending on the composition and structure of the starting substances, different mixtures of hydrocarbons were formed in which all of the typical representatives of petroleum were present, but in different proportions.

The investigation of contemporary and Late Quaternary marine sediments showed that, in certain phases of these depositions, the reduction of the bituminous (lipoidal) part of the organic matter of the sediment occurs, and it approaches petroleum in its chemical composition as hydrocarbons of the petroleum type accumulate in it (Ginzburg-Karagicheva, Pryanishnikov and Rodionova, 1934; Veber and Gorskaya, 1950). The transformation of the lipoidal components of the sediment begins from the moment of its deposition and, according to Veber's data (1958), continues to depths of 200-400 m (in a cross section of the ancient Caspian), and according to Smith's data (1956), to a depth of about 100 m (Pelican Island). According to Veber (1958), the process of the formation of petroleum hydrocarbons in the system of geological cycles occurs at the stage of diagenesis of sediments. Levorsen (Levorsen et al., 1956) holds to a similar view.

The participation of microorganisms in the conversion of the organic matter of contemporary marine sediments has been confirmed by a large number of works by Russian and foreign investigators, who have shown the presence of a varied microflora in marine organogenic silts. The greatest number of microorganisms is concentrated in the surface layers of the sediments; however, the presence of active heterotrophic bacteria has been established even at depths of 22.5-34 m from the surface of the sea bottom (Messineva, 1956; Mekhtieva, 1956). Microorganisms are active converters of the overwhelming majority of organic substances, including lipoids.

According to Bach and Sierp's assumptions (1924), fatty acids which are released during the hydrolysis of fats can be converted to hydrocarbons by splitting off CO_2. They came to this conclusion on the basis of data on the decrease in the saponification number and the increase of the iodine number of fat. Ginzburg-Karagicheva et al. (1934) arrived at similar views. In a number of works, Seliber (1926, 1931, 1945, 1950, 1928) stated the hypothesis of the possible transformation of fats to petroleum hydrocarbons through the action of sulfate-reducing bacteria. However, direct decarboxylation of high-molecular fatty acids by the action of microorganisms was observed only in the experiments of Ginzburg-Karagicheva et al. (1934) and Laigret (1949) with oleic acid.

We set ourselves the task of studying the character of the conversion of fats under aerobic and anaerobic conditions by the action of aboriginal microorganisms of rocks and petroleums and contemporary marine organogenic silts.

In distinction from the majority of the investigators working with sulfate-reducing or fat-splitting* microorganisms, we concentrated on the denitrifying group of bacteria which is widely distributed in both Tertiary petroleums and rocks and in contemporary marine sediments (Ginzburg-Karagicheva, 1936; Reinfel'd, 1936; Maliyants, 1939; Shturm, 1950; Kolesnik, 1955; Mekhtieva, 1956; Mekhtieva and Malkova, 1958; Rybakova, 1957).

The decomposition of lipoids in the course of denitrification has been studied very little, although this process is widely developed in the upper layer of subaquatic and subaerial sediments enriched with organic substances and nitrates.

EXPERIMENTAL PART

We isolated more than 20 pure cultures of denitrifying bacteria from Tertiary petroleums in Apsheron and from the argillo-siltstone strata of Tertiary age in North Caucasian Valley. These cultures were used to set up experiments on the conversion of liquid and solid fats of various origins--beef fat, sunflower oil, dolphin, fish, and plankton fats--under aerobic and anaerobic conditions.

In the present communication, data are presented on the first series of experiments, i.e., on the conversion of beef fat and sunflower oil in a solution of mineral salts† with the access of air. The concentration of NaCl in this solution constituted 30‰, which approximates the content of this salt in ocean water.

* The division into "fat-splitting" (lipolytic) and sulfate-reducing bacteria is quite conditional; we are using these terms, as well as the term "denitrifying" on the strength of established tradition, since they give some idea of the conditions of conducting the experiments.

† KNO_3—2 g, $MgSO_4$—2 g, K_2HPO_4—2 g, $CaCl_2$—0.2 g, NaCl—30 g, $FeSO_4$—traces, distilled water—1 liter, solution of microelements (according to Meiklejohn)—1 ml.

The fat was boiled repeatedly with distilled water prior to the experiment in order to remove traces of water-soluble nitrogenous substances; oil was not subjected to any preliminary treatment. The fats being tested were purposely not refined in order to avoid the breakdown of the phospholipids included in their composition.

The test cultures were grown at 30 deg for 6 months. Fifteen g of fat and 200 ml of salt solution were added to each vessel (flat-bottomed flask) measuring 250 ml. Solid fat was first melted slightly and was spread evenly over the bottom and walls of the flask. The flask was covered with a cotton plug and a double parchment cap.

Visual observations showed that the denitrification process went on very vigorously even in the first days, with violent gas formation. After a week, the medium became very turbid, and the outward appearance of the fat being tested changed markedly: solid fat disintegrated and acquired a pasty consistency, while liquid fat became turbid and white flakes formed on the margin of its contact with the solution.

In control vessels, the fat hardly changed at all throughout the entire period of the experiments; the medium remained transparent. During the course of the experiment, a 1% KNO_3 solution was added to the vessels (in 5 ml amounts) 1-3 times as the nitrates disappeared from the medium.

After 6 months, the contents of the experimental flasks with solid fat were filtered through a paper filter; the fat remaining in the flask and the filter with fat were dried for 1 hour under vacuum at 35 deg.

The dried filter was transferred to a culture flask and was treated three times with petroleum ether (boiling point 45 deg) while heating with a reflux condenser on a water bath. The hot ether solution was filtered through a paper filter, dried with anhydrous Na_2SO_4, and distilled off into a weighed flask in a stream of carbon dioxide.

Liquid fat was separated from the aqueous medium by means of a separatory funnel and was extracted with petroleum ether. In view of the fact that it is easily soluble, heat was not used. The fat isolated from the solution was dried under vacuum in a drying cabinet at 35 deg for 2 hours, and then for 24 hours in a desiccator over calcium chloride, after which its iodine and acid numbers, saponification number, and non-saponifiable residue were determined.

The residue remaining after the extraction of fat was treated by heating with 10% H_2SO_4. The extracted fatty acids were separated from the aqueous layer in a separatory funnel, washed with distilled water till the disappearance of the acid reaction, and dried to constant weight in a desiccator. In the fatty acids, the neutralization number and molecular weight were determined. It should be noted that the presence of an ether-insoluble residue (consisting principally of calcium and magnesium soaps of saturated high-molecular fatty acids) was noted only in solid fat. After filtration, the culture fluid was acidified with 10% HCl to split the soluble (potassium and sodium) salts of high-molecular fatty acids which might be present in the solution. However, the amount of fatty acids extracted in this case was so insignificant, that it was impossible to collect them. The fat from 84 experiments was analyzed.

The analysis of fat was carried out according to the usual methodology which is given in detail in the practical manuals on biochemistry by Kizel' (1934), Belozerskii and Proskuryakov (1951), and Ermakov and coworkers (1952).

In the overwhelming majority of experiments with solid fat (Fig. 1), there was a considerable increase

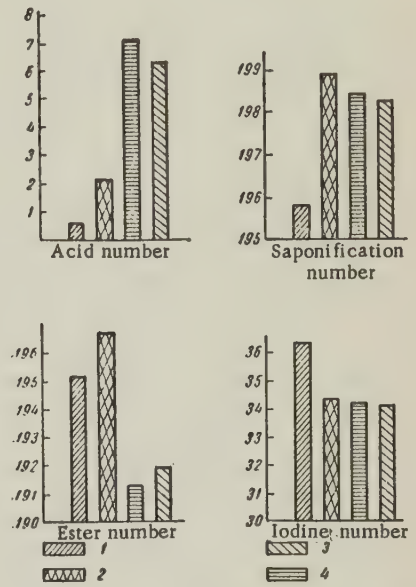

Fig. 1. Average values of the chemical constant of animal fat: 1) Original fat; 2) control; 3) bacteria isolated from stratum; 4) bacteria isolated from petroleum.

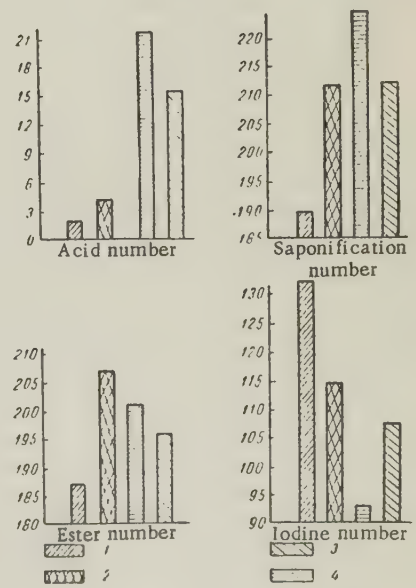

Fig. 2. Average values of the chemical constants of vegetable oil: designations same as in Fig. 1.

(in comparison with the control) in the acid number, which is evidence of the accumulation of free fatty acids. The sharp reduction of the ester number indicates the splitting of ester bonds. The saponification number and the iodine number showed essentially no change in comparison with the sterile control. From the flaky residue which was insoluble in petroleum ether, considerable amounts of fatty acids (from 0.37 to 2.06 g) with molecular weights of from 252.2 to 267.9 were isolated following treatment with 10% H_2SO_4.

In experiments with vegetable oil (Fig. 2), an increase in the acid number and a decrease in the ester number were also noted and, in distinction from solid fat, a sharp decrease in the iodine number connected with the saturation of the double bonds.

It should be noted that when sterile fat was kept in weakly alkaline aqueous medium for a long time, a considerable chemical alteration followed, which was indicated by the reduction of the iodine number and by increases in the acid number, ester number, and saponification number, which somewhat obscured the results of the bacterial effect on the fat. Nevertheless, the average data represented in Figs. 1 and 2 are evidence of the fact that solid and liquid fats were subjected to intensive bacterial action, which resulted in the splitting of the ester bonds of the triglycerides of fatty acids and in the accumulation of fatty acids in the free form and in the form of soaps.

A simple experiment showed that the test microorganisms chiefly utilize glycerol as a source of organic carbon under experimental conditions, and fatty acids only to an insignificant degree.

The cultures used in the present series of experiments were inoculated into test tubes of the original mineral medium with various sources of organic carbon: glycerol, oleic, stearic, and palmitic acids. In the overwhelming majority of cultures, nitrate reduction went on very vigorously in the presence of glycerol and rather weakly (and unequally in different cultures) in the presence of fatty acids. The results of the investigation of the nonsaponifiable residue will be given in the second communication.

DISCUSSION OF RESULTS

The experiments conducted suggest that denitrifying bacteria isolated from rocks and petroleums of Tertiary age exhibit strong lipolytic properties; they utilize animal and vegetable fats in the process of energy and synthetic metabolism, at the same time reducing nitrates to gaseous nitrogen.

The process of fat decomposition under the influence of these microorganisms starts with hydrolysis. The splitting of ester bonds occurs as the result of the action of bacterial lipases; the glycerol and fatty acids released undergo further conversions which proceed differently depending on the specificity of the biochemical activity of the particular microbial forms.

In this case, the hydrolysis products of fats are utilized unequally by the bacteria: glycerol is oxidized very intensively, while fatty acids—rather poorly. The presence of nitrate salts in sea water, as well as the presence of a large number of denitrifying forms in contemporary sediments, makes it reasonable to suppose that the conversion of organic substances, including lipoids, in the top layer of the sediment occurs with the active participation of denitrifying bacteria. The process of denitrification in the presence of fats goes on very intensively. It is natural to suppose that the lipoids present in organogenic silts are first subjected to the action of denitrifying bacteria which grow over a very wide range of oxidation-reduction potential and at different partial pressures of oxygen. As the result of the vital activity of these (as well as of other) microbial forms, the medium acquires a strongly reduced character which favors the growth of anaerobic sulfate-reducing bacteria which, along with other substances, also utilize fatty acids which are poorly accessible to the action of lipolytic denitrifying bacteria.

SUMMARY

1. A study has been made of the conversion of fats of animal and plant origin by the action of denitrifying bacteria in aqueous medium with access of air, i.e., under conditions similar to those produced in marine organogenic silts.

2. It has been established that fats undergo extensive hydrolysis under the influence of denitrifying bacteria; ester bonds are split, and glycerol and fatty acids undergo bacterial oxidation at the expense of the oxygen of nitrates. The acid number of the test fat rises sharply due to the formation of free fatty acids; the iodine number decreases as the result of the saturation of double bonds.

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INDUCED AMYLASE SYNTHESIS IN ASPERGILLUS ORYZAE MYCELIUM

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It is well known that the enzymatic properties of certain microorganisms are manifested almost immediately after the cells are brought into contact with the specific substrate.

In other cases, a more or less prolonged induction period is required before the activity of the enzyme is revealed. It is precisely on the basis of this fact that, in 1930, Karstrom proposed that enzymes be divided into adaptive and constitutive ones. Aside from the length of the lag period, the criterion for this type of grouping of enzymes was the degree of increase of enzyme synthesis in response to the addition of the specific substrate. If the amount of enzyme increased at least five times, Karstrom placed it in the adaptive group; otherwise, it was grouped with the constitutive enzymes (Karstrom, 1938).

In the preceding work (Tikhomirova, 1959), we compared the amount of amylase, dextrinase, and maltase produced during the growth of Aspergillus oryzae on media with various carbon sources; at the same time, the stimulation of the synthesis of amylolytic enzymes by specific carbon sources and by their hydrolysis products was noted. These observations made it possible to place the amylolytic enzymes of A. oryzae in the adaptive group of enzymes.

The purpose of the present investigation was to confirm the adaptive formation of amylase in A. oryzae by means of Karstrom's classical methods (Karstrom, 1930, 1938). For this purpose, observations were carried out on the duration of the lag period during which the fungal mycelium, grown on medium containing a carbon source which is nonspecific for amylase and then transferred to a starch solution, does not increase amylase synthesis. In addition to this, the amount of amylase formed as the result of keeping the mycelium on starch solutions was compared with that formed on substrate which is nonspecific for amylase.

METHODS

A. oryzae 153 was grown on a shaker at 30 deg for three days. The composition of the medium was as follows (in %): glycerol—4.5; $(NH_4)_2SO_4$—0.69 (0.15% by N); KCl—0.05; $MgSO_4$—0.05; KH_2PO_4—0.1; $FeSO_4$—0.001; $CaCO_3$—0.5; distilled water—100 ml; pH 6.8-7.

Glycerol was used as the carbon source, since good growth of the fungus on glycerol-containing media was accompanied by minimal accumulation of amylolytic enzymes (Tikhomirova, 1959). The fungal mycelium was filtered off, washed with three volumes of 1/15 M phosphate buffer (pH 6), and resuspended in a small amount of the same buffer. In one of the identical samples of mycelium, the initial amount of amylase was determined, while in another—the dry weight of the mycelium; the remaining samples were placed in a series of flasks with 50 ml of phosphate buffer containing 0.5% of soluble starch and all the mineral components of the medium with the exception of $(NH_4)_2SO_4$ and $CaCO_3$. The mycelium was aerated on a shaker at 30 deg; after certain intervals of time, amylase was determined in the mycelium and in the solution. The amylase was accounted for as the saccharifying capacity (SC) of the enzyme. The amount of enzyme which produces 1 mg of maltose from a 1% starch solution buffered to pH 4.7 in 1 hour at 30 deg was taken as one SC unit. The techniques of the analysis and the calculations were given in the preceding work (Tikhomirova, 1959).

EXPERIMENTAL

In the first series of experiments, the washed mycelium of A. oryzae 153, grown on medium where glycerol was the sole carbon source and where the amount of amylase was insignificant, was transferred to phosphate buffer (pH 6) containing 0.5% starch. All of the salts included in the nutrient medium except $(NH_4)_2SO_4$ and $CaCO_3$ were added to the starch solution. Under such conditions the fungus increased amylase synthesis, not immediately after contact with the starch, but after an interval of one hour. From the first to the second hour, the enzyme accumulated very slowly. Only from the second hour of growth on starch did the rate of enzyme production rise proportionally to the induction time, while after the sixth hour, the rate of synthesis became constant (Table 1).

In control experiments, where the washed mycelium was kept in a solution in which the starch was replaced by glycerol, 20.3 times less amylase accumulated in 8 hours (on glycerol, it increased from 66 SC units to 111 units, on starch—to 913 SC units).

The presence of an hour-long lag period during the synthesis of amylase on starch, and the sharp rise in enzyme production in response to the addition of the specific substrate, once again convincingly confirmed the conclusion made earlier (Tikhomirova, 1959) concerning the adaptive character of A. oryzae amylase.

In the initial experiments on induced amylase synthesis, the concentration of starch used was 0.5%. Later, in special experiments, the dependence of amylase synthesis on the concentration of specific substrate was established. For this, identical samples of mycelium were transferred to starch solutions of different concentrations—0.1; 0.3; 0.5; 0.7; 0.9%—and were aerated on a shaker for four hours. Enzyme synthesis proceeded uniformly in all the variants tested; moreover, there was even somewhat more amylase formed on 0.1% starch (Table 2). Despite the more active formation of amylase at the inducer concentration of 0.1%, the length of the lag phase during incuded enzyme syn-

Table 1. Amylase Synthesis by A. oryzae Mycelium Grown on Glycerol-Containing Medium and Transferred to 0.5% Starch Solution, pH 6 (average of 2 experiments)

Duration of keeping mycelium on starch, hours	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
0	0.799	0	55	55	0	66	66
0.25	0.775	0	30	30	0	40	40
1	0.770	0	47	47	0	60	60
2	0.823	14	60	74	17	74	91
3	0.857	53	218	271	62	255	317
4	0.857	314	262	576	366	303	673
5	0.857	386	248	634	450	289	754
6	0.857	510	256	766	595	298	893
7	0.865	485	254	739	560	293	853
8	0.857	506	277	783	590	323	913
Control: 8 hours—on glycerol	0.964	62	41	103	63	48	111

Table 2. The Effect of Various Starch Concentrations on Amylase Synthesis by A. oryzae Mycelium. Length of Induction—4 Hours, pH 6 (average of 5 experiments)

Starch concentration, %	Dry weight of mycelium	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
0.1	0.976	356	269	625	329	276	638
0.3	1.006	305	268	573	310	273	583
0.5	1.006	260	259	519	243	265	508
0.7	1.006	235	255	490	240	259	499
0.9	1.006	282	259	542	285	263	548

thesis in the starch solution of the given concentration did not decrease in comparison with the lag phase observed in 0.5% starch (Table 3).

An important biological factor determining the vital activity of the cell is the active acidity of the medium in which the microorganism is placed. It is known that the optimal pH value for the activity of fungal amylase lies within the range of 4.5-5.0. The pH value which is best for amylase synthesis has not been established precisely. Therefore, a series of experiments was conducted in which 0.1% starch solution was adjusted to the following pH values: 3, 4, 5, 6, 7, 8, 9, 10. A neutral reaction of the medium proved to be optimal for amylase synthesis by A. oryzae. The greatest per cent of amylase given off from the mycelium into the fluid was also observed in the starch solution with a pH of 7 (Table 4).

A 0.1% starch solution made with pH 7 phosphate buffer was used in all further experiments.

Although aspergilli belong to the auxoautotrophs, and even though amylase is a simple enzyme which does not contain a nonprotein part, the effect of vitamins on the induced synthesis of amylase was still checked in the experiments. In performing such experiments, the aim was to stimulate amylase production by means of adding ready-made parts of other enzymes participating in the synthesis of amylase itself. Of all the vitamins tested (Table 5), only pyridoxine gave some increase in amylase synthesis. The stimulation of amylase production by pyridoxine is entirely according to pattern, since the given vitamin is a constituent part of enzymes responsible for the transamination of amino acids. At present, the synthesis of adaptive enzymes is associated with the de novo formation of protein molecules or with the conversion of proteins already existing in the cell.

Spiegelman and Halvorson (1956) established that the presence of a nitrogen source in the medium affects

Table 3. Amylase Synthesis by A. oryzae Mycelium Grown on Medium Containing Glycerol and Transferred to a 0.1% Starch Solution, pH 6 (average of 2-3 experiments)

Duration of keeping mycelium on starch, hours	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
0	0.765	0	59	59	0	77	77
0.25	0.765	0	39	39	0	52	52
0.75	0.765	0	64	64	0	83	83
1	0.765	0	64	64	0	83	83
1.5	0.765	0	86	86	0	112	112
2	0.765	0	103	103	0	134	134
2.5	0.822	88	240	329	104	282	386
3	0.855	115	243	358	131	272	403
4	0.821	439	251	690	494	305	799
5	0.821	535	262	797	659	319	978
6	0.821	581	255	885	772	620	1082
7	0.821	613	259	872	751	316	1067
8	0.821	688	233	911	841	314	1155

Table 4. Effect of the pH of a 0.1% Starch Solution on the Adaptive Synthesis of Amylase by A. oryzae Mycelium. Length of Induction—4 Hours (average of 3 experiments)

Initial pH of starch solution	Final pH of starch solution	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
			In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
3	5.6	0.714	31	718	749	44 = 4%	1013 = 96%	1057
4	5.9	0.714	21	630	651	30 = 3%	896 = 97%	926
5	6.0	0.714	73	819	892	102 = 8%	1138 = 92%	1240
6	6.2	0.714	274	778	1052	389 = 27%	1080 = 73%	1469
7	7.2	0.714	591	673	1264	827 = 46%	946 = 54%	1773
8	7.9	0.714	392	542	934	553 = 42%	764 = 58%	1321
9	8.0	0.714	387	504	891	546 = 43%	713 = 57%	1259
10	8.0	0.714	249	393	642	336 = 39%	550 = 61%	866

the synthesis of adaptive enzymes, since without nitrogen, the synthesis of the protein molecule is hampered. This rule was also confirmed in our experiments. Thus, washed A. oryzae mycelium grown on medium with glycerol and then transferred to a starch solution containing nitrogen in the form of ammonium sulfate and without nitrogen, showed a 1.5-fold increase in amylase synthesis in the presence of 0.05% nitrogen as compared with the nitrogen-free medium (Table 6). In order to avoid an initial excess of nitrogen in the fungal cells, the amount of nitrogen in the medium for growing the fungus was decreased by 1/3 in these and subsequent experiments. It was previously established that such a decrease in nitrogen does not reduce the rate of amylase synthesis by mycelium transferred to starch.

The experiments presented above demonstrated the importance of the presence of nitrogen in the medium for the synthesis of the adaptive enzyme. It was interesting to determine whether amylase synthesis would increase if the fungus would have nitrogen available to it in the form of ready-made constituent parts of amylase rather than as mineral nitrogen. For this purpose, various amounts of highly purified amylase preparation from A. oryzae which had been heat-inactivated were added to 50 ml of the 0.1% starch inducer. The largest amounts of amylase were formed in the presence of 0.05% of the given preparation, but these values were no higher than in medium containing 0.5% nitrogen in the form of ammonium sulfate (Table 7). From the experimental data obtained, it can be concluded that enzyme synthesis in the presence of ready-made constituents of amylase does not differ qualitatively from the synthesis of the given protein from mineral nitrogen. However, the present experiments can only be regarded as preliminary, since we

Table 5. The Effect of Vitamins on Induced Amylase Synthesis by A. oryzae Mycelium. Length of Induction—4 Hours, pH 7 (average of 2-3 experiments)

Name of vitamin added to starch solution	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
Without vitamins	0.577	212	110	322	365	193	559
Biotin (5 γ per 1 liter)	0.671	271	112	383	421	157	578
Thiamine (100 γ per 1 liter)	0.671	263	108	371	404	150	554
Pantothenic acid (1000 γ per 1 liter)	0.671	254	111	365	394	155	548
Pyridoxine (100 per 1 liter)	0.577	340	127	467	393	232	625
Riboflavin (1000 γ per 1 liter)	0.763	212	178	390	316	232	538
Nicotinic acid (1000 per 1 liter)	0.671	279	114	393	430	157	587
Inositol (4000 γ per 1 liter)	0.572	228	111	339	396	193	589

Table 6. The Effect of Various Amounts of Nitrogen on Induced Amylase Synthesis by A. oryzae Mycelium. Length of Induction—4 Hours, pH 7 (average of 3 experiments)

Amount of nitrogen in the form of $(NH_4)_2SO_4$ added to starch (% N)	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
Without N	0.518	168	151	319	312	306	618
0.0025	0.625	219	169	388	351	271	622
0.005	0.580	220	146	366	382	252	634
0.006	0.580	232	157	389	402	270	672
0.008	0.580	228	153	381	393	265	658
0.0125	0.576	121	142	263	375	312	687
0.025	0.576	195	143	338	398	306	704
0.05	0.576	253	252	505	462	466	928
0.15	0.576	247	244	491	439	442	881

did not have a crystalline preparation of amylase at our disposal.

Is the adaptive formation of amylase in the absence of exogenous nitrogen connected with the growth of fungal mycelium or does it occur in nongrowing cells as well? The possibility of the induced synthesis of A. oryzae amylase in the absence of growth could be graphically demonstrated by keeping the fungal mycelium in 50 ml of starch solution to which 3 ml of toluene was added. In experiments with toluene, no accumulation of amylase was observed during an interval of 11 hours. In the rest of the experiments, adaptive amylase synthesis was accompanied by growth of the fungal mycelium, even in nitrogen-free medium with starch. (The amount of mycelium increased after keeping it for four hours on starch with 0.583 g to 0.642 g.)

The experiments on the induced synthesis of amylase show that the fungus carries out the synthesis of new enzyme protein both in the presence and the absence of an exogenous nitrogen source. During the process of induction in nitrogen-free medium, the nitrogen used by the cell for the synthesis of the new enzyme molecule is evidently abstracted from the nitrogenous compounds present in the cells. A rearrangement of nitrogenous substances takes place inside the cell. The protein molecules of amylase accumulate, despite the relative impoverishment of the fungal cells with respect to nitrogen which takes place as the result of the con-

Table 7. The Effect of Inactivated Amylase Preparation on Induced Amylase Synthesis by A. oryzae Mycelium. Length of Induction—4 Hours, pH 7 (average of 3 experiments)

Amount of inactivated amylase preparation added to starch solution	Dry weight of mycelium, g	Total amount of amylase (in SC units)			Amount of amylase recalculated for 1 g of dry mycelium (in SC units)		
		In 50 ml of solution	In total mycelium	In solution and in mycelium	In 50 ml of solution	In mycelium	In solution and in mycelium
Without amylase preparation, without $(NH_4)_2SO_4$	0.584	220	159	379	377	272	649
0.05% N in the form of $(NH_4)_2SO_4$, without preparation	0.584	245	223	468	419	283	702
0.05% preparation, without $(NH_4)_2SO_4$	0.584	206	180	386	352	309	661
0.05% preparation, without $(NH_4)_2SO_4$	0.584	217	190	407	373	325	698
0.1% preparation, without $(NH_4)_2SO_4$	0.584	187	149	336	321	255	576
0.5% preparation, without $(NH_4)_2SO_4$	0.536	133	109	242	248	203	451
1.5% preparation, without $(NH_4)_2SO_4$	0.536	72	36	108	133	68	201

tinuing increment in the mycelial biomass in medium lacking exogenous nitrogen.

In conclusion, I should like to offer deep gratitude to Prof. A. A. Imshenetskii for directing the given work.

SUMMARY

1. The dynamics of induced amylase synthesis by washed Aspergillus oryzae mycelium was studied; the fungus was grown in medium containing glycerol as the sole carbon source and was transferred to a starch solution.

2. The presence of an hour-long lag period during the induction of amylase by starch is evidence of the adaptive character of amylase in the fungal species under investigation.

3. A 20-fold increase in amylase synthesis, when the mycelium is kept on specific substrate as compared with synthesis on glycerol, confirms the adaptive character of amylase.

4. The amount of enzyme is not proportional to the concentration of the inducer. Amylase is formed most intensively in 0.1% starch solution.

5. A neutral reaction of the medium (pH 7) is optimal for the induced synthesis of A. oryzae amylase.

6. The presence of vitamins--thiamine, biotin, pantothenic acid, riboflavin, nicotinic acid, inositol--in the inducer solution does not increase amylase synthesis. Only pyridoxine has some stimulatory effect on this process.

7. The presence of 0.05% mineral nitrogen in the starch solution in the form of ammonium sulfate increases amylase induction 1.5 times.

8. Toluene inhibits induced amylase synthesis.

9. The adaptive synthesis of amylase in starch solution without an exogenous nitrogen source is accompanied by an increment in the mycelial mass of the fungus.

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PECIES COMPOSITION OF THE EPIPHYTIC MICROFLORA OF LIVING PLANTS

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The green parts of growing plants are abundantly ıhabited by microflora. This was reported as early s 1903 by Burri. This author isolated the predomiant species which proved to be the following: Bacerium herbicola, B. fluorescens, B. putidum. Duggeli 904), who continued Burri's work, proved that the ource of the epiphytic microflora of plants is not the ir, but rather the seeds and partly the soil.

Later, not as much attention was devoted to the tudy of the epiphytic microflora. The work was cared out chiefly along the following lines: 1) Its effect n milk was determined when it got in from feeds; the effect of the epiphytic microflora on processes ccurring during the ensilaging of the green mass of ants was studied; 3) its role in the process of grain torage was studied in relation to moisture, spontneous heating, etc.; 4) the role of the epiphytic icroflora of seeds in the formation of root and hizosphere microorganism complexes was deterined. In the majority of cases, the species compotion was studied incidentally during the solution of ther problems, and therefore, was not studied thorughly enough. Nevertheless, in all works we find formation concerning the qualitative composition of e epiphytic microflora of the green mass or of the rain.

Starygina (1926), while investigating the microflora feeds (in connection with the possibility of its getng into milk), found B. herbicola and B. fluorescens be predominant in 17 of the 33 samples of herbaeous plants analyzed. Sporogenous microorganisms, e wild yeast–oidium, and actinomycetes predomiated on woody plants. Woller (1929) confirmed the ata on the migration of bacteria from seeds to seedngs and described B. herbicola and fluorescent and porogenous forms as the predominant species. He metimes encountered B. coli, B. punctatum, and ctic acid bacteria in large numbers.

The predominance of B. herbicola and B. fluoescens on plants was pointed out by Mack (1936) d by other authors studying the microflora of the silaged mass (Mishustin, 1933; Makarova and Gavlova, 1935; Zubrilin et al., 1950). In addition, B. esentericus, B. megatherium, micrococci, and sarnas were encountered in small numbers.

Kvasnikov and Sumnevich (1953) found many pigented forms, yeasts, B. coli, ammonifiers, and buric acid bacteria on plants. There were more lactic id microorganisms in the rhizosphere.

The composition of the epiphytic microflora of seeds es not differ significantly from the composition of e microflora of the green parts of plants.

Hofer and Hamilton (1940) showed that the composition of the microflora of the different members of the grass family is quite similar.

The microflora of seeds can affect the processes occurring during their storage. From this point of view, its compositions has interested a number of investigators, who found B. herbicola to be predominant on wheat (Rautenshtein, 1939; Pod'yapol'skaya and Mirzoeva, 1955) and on millet (Romakina, 1949).

The work of Medvinskaya and Rozhdestvenskii (1948) was devoted to the presence of sporogenous bacteria on grain. When wheat was being harvested, sporogenous microorganisms were found on the grain regularly, but in small numbers (10-100 per 1 g of grain). Among the bacteria found were: B. herbicola, B. coli luteoliquefaciens, B. fluorescens, and Proteus vulgaris.

Sorokina (1958) studied the epiphytic microflora of kok-saghyz seeds. In the author's opinion, its main representatives are P. herbicola, Chromob. flavum, and Mycob. phlei. P. radiobacter and P. fluorescens are encountered in smaller numbers.

At present, there are indications in the literature that when seeds germinate, the epiphytic microflora passes not only to the leaves, but to the roots as well, i.e., the microflora of the root system and the plant rhizosphere is formed with the participation of the epiphytic microflora of the seeds (Wallace and Lochhead, 1951; Khudyakov and Voznyakovskaya, 1956, Kvasnikov, 1957; Petrenko, 1958; Fedorov and Pantosh, 1952).

At the present time, it has still not been determined which species of the entire population of the root microflora belong to the true epiphytes.

Investigators have found a relatively small number of species on the green parts of plants and on seeds, and B. herbicola and P. fluorescens always predominated. It is important to note that almost all investigators used meat-peptone agar as the nutrient medium for isolating epiphytic microorganisms, and, consequently, the study of this question has progressed little in comparison with the classical investigations of Burri and Duggeli. Also, in connection with this, the necessity of controlling the composition of this microflora did not arise, since it was distinguished by little variety.

It has now been established with certainty that the composition of the nutrient medium is of decisive significance in determining the qualitative range of microorganisms which can be isolated from any plant substrate.

Actually, Khudyakov (1953) found 20 species of epiphytic microorganisms on hay decoction. Khudyakov and Voznyakovskaya (1956) obtained an even greater variety while isolating the root and epiphytic micro-

flora of wheat; they used a nutrient medium made of cabbage decoction for this purpose. A total of 41 species and 23 varieties of the latter were isolated.

Under natural conditions, the epiphytic microflora lives at the expense of nutrient substances secreted through the seed membrane or the surfaces of leaves and stems as the result of exosmosis and by the process of guttation. The composition of the substances secreted by seeds was studied by Borner (1955), who used the chromatography method. The author showed that the seeds of cereal grasses, both during swelling and without swelling, secrete five amino acids (asparagine, glutamine, alanine, valine, and leucine) and two kinds of sugars (glucose and xylose).

Chibnall (1939) detected glutamine in the guttation droplets of rye grass.

EXPERIMENTAL

On the basis of the data presented, a cabbage medium was used in the present work which was enriched with amino acids by means of adding 25 ml of wort and 1.25 ml of corn extract to it (the so-called medium No. 19). Its utilization as a basic medium for the isolation of epiphytic microflora was reported in the work of Voznyakovskaya and Shirokov (1958).

For comparison, we inoculated the same plant sample into medium No. 19 and MPA (Table 1). These results confirmed that it is not expedient to use meat-peptone agar for studying the species composition of the epiphytic microflora.

Table 1. Inoculation of the Microorganisms Washed From Leaves on Medium No. 19 and on MPA

Leaves	Species growing up	
	On medium No. 19	On MPA
Winter wheat	14	4
Birch	6	0
Oats	6	2
Potato	10	4

We collected the plant samples for isolating epiphytic microflora in fields, in the woods, and on the plantations of the fruit and berry experimental station in the Lenin District of the Moscow Region; grape leaves were collected in Crimea. A total of 18 plant species was investigated: winter wheat, oats, corn, buckwheat, rye grass, flax, sunflower, pea, clover, potato, garden beet, raspberry, gooseberry, black currant, grape, apple, oak, and pine. In cereal grasses and leguminous plants, microorganisms were isolated from the stems and leaves; in vegetable cultures—from leaves; in shrubs and tree species—from leaves and needles.

The samples for analysis were made up as averages of five samples of each plant species collected from different places in the field, woods, or garden. A weighed portion of 5 g was placed in a flask with 100 ml of sterile water and was shaken vigorously for 10 minutes; then, 1 ml of the washings was transferred from the flask to a test tube containing 10 ml of water. One drop of the resulting dilution was inoculated on the surface of agar (medium 19) poured into a Petri dish. The drop was spread with a spatula on three successive plates. All of the different colonies which grew up were subcultured on agar slants in test tubes, were checked for purity and, after their morphology and physiological properties were studied, were identified according to Krasil'nikov's key (1949).

In order to have some idea of the predominance of particular species on different plants, a quantitative colony count was made.

When the inoculated plates were examined superficially, it was striking that pigmented forms inhabit the green parts of plants in large numbers. After determining the species composition of 165 cultures isolated from 2747 colonies, it was found that they belong to 47 different species. Frequently, representatives not of the main species, but of some of the varieties included in it were encountered, or cultures in which some of the properties deviated from those described in the key. Thirty-six such cultures were found. With respect to colony color, of 47 species, 8 yellow-pigmented ones were found, 8 lemon-colored, 13 orange and red, 6 white, 2 brown, and the remaining 10—colorless.

All of the representatives of the epiphytic microflora isolated were grouped by genera in the following manner: Pseudomonas—11; Bacterium—3; Chromobacterium—5; Mycobacterium—9; Pseudobacterium—3; Micrococcus and Mycococcus—6; Lactobacterium—1; yeasts—4; Bacillus—2; Sarcine—3. Thus, Pseudomonas and Mycobacterium proved to be predominant; Chromobacterium and Micrococcus were also encountered in considerable numbers.

For a more graphic idea of the epiphytic microflora, we present a brief description of the outward appearance of the colonies of the different epiphyte species grown on medium No. 19. For a simpler and more convenient orientation with regard to the whole variety of microorganisms, we considered it expedient to group them according to colony color. These data are given in Table 2.

The determination and enumeration of the species of epiphytic microorganisms which are encountered on the green parts of plants still give no idea of which of them predominate and whether or not there are differences in the composition of the epiphytic microflora on different species of plants. If those species which are encountered in a number of cases in amounts of 10 or more per cent of the total number of microorganisms found on plants are taken as predominant species, it will be found that 21 of the 47 will belong to such species, namely the following: P. herbicola, P. fluorescens, P. liquefaciens, P. glycines, P. resinaseae, P. epiphyticum n. sp., Ps. not identified, B. album, C. aurantiaca, C. flavum, C. flavascens, M. mucosum, M. citreum, M. album, P. lacticum, M. candidus, M. luteus, M. luteus, S. flava, mycelial yeasts, B. megatherium. Of these, simultaneous predominance on several species of plants was found for the following 10 species: P. herbicola, P. fluorescens, P. glycines, P. resinaceae, Ps. not identified, M. al-

Table 2. Character of Growth of Epiphytic Microorganisms When Isolated on Cabbage Agar

Colony color	Species	Variety	Outward appearance of colony on cabbage (improved) medium
Yellow	Pseudomonas herbicola	trifolii, agglomerans	Yellow, quite slimy, not convex. In agglomerans, border is lighter-colored
	Pseudomonas glycines	Decompose gelatin and starch weakly	Pale yellow, transparent, flat, sometimes with a yellower center
	Chromobact. flavum	rigens	Yellow, transparent, flat with well-defined border
	Chromobact. rheni	harrisonii	Dark-yellow, quite convex, shiny
	Chromobact. flavescens	According to Bergey	Yellow, convex, small, transparent
	Mycobact. flavum	–	From cream to orange, flat, transparent
	Pseudobact. flavum	–	Yellowish, slightly slimy, medium size
	Bac. megaterium	Different with respect to changes in gelatin, milk, and starch	Cream, not transparent, shiny
Lemon	Pseudomonas turcosa	–	Pale lemon, slimy, convex
	Myc. citreum	vulgatum	Greenish-milky, not transparent, pasty, shiny
		liquefaciens	Greenish-yellow, flat-convex, transparent
	Pseudobact. lacticum	Does not reduce NO_3	At first, colonies small, greenish, then flat-convex
		Decomposes starch	Plaques of medium size with darker center, transparent
	Micr. citreus	plantarum	Pistachio-colored, not transparent, flat-convex, shiny
		cerinus	Greenish-milky, convex, pasty
	Micr. luteus	–	Whitish at first, then becomes green, convex, not transparent, small
	Mycoc. luteus	–	Lemon-milky, shiny, large
	Sarcina letea	–	Greenish-yellow, not large, frequently with uneven surface, not transparent, pasty, with waxy gloss
	Sarcina flava	–	Lemon, quite large, waxy gloss, not transparent, frequently with raised center
Orange and reddish	Ps. resinacea	Does not curdle milk	Bright-orange, convex, transparent, shiny
	Ps. carnea	–	Pinkish-orange, flat, shiny, medium size
	Chromob. aurantiaca	plicatum	Yellow-orange, flat, thick slime, comes off as a film
		ractiata	Orange, quite large, flat, pasty, rather dry
	Chr. rubidum	–	Brownish-orange, slimy, convex
	Mycobact. mucosum	perrugineum	Pinkish-cream, very slimy or pasty, quite flat
	Myc. phlei	–	Orange, convex, semi-slimy, not large
	Myc. rubrum	equi	Bright orange, convex, shiny, not transparent
	Myc. brevicale	–	Pinkish-orange, frequently with markings, not transparent, flat-convex
Orange and reddish	Pseudobact. rubricum	rufum	Pale pink, small, glassy, convex
	Micr. aurantiaca	rubescens	Pink, large, slimy, not transparent
	Mycoc. ruber	–	Dark pink with darker center, quite flat, shiny, not large
	Planosarc. agilis	–	Orange, convex or flat, shiny, transparent
	Torula rosea	–	Bright pink, convex, not transparent, shiny, pasty
White	Bact. album	guttatum	Whitish, small, shiny
	Bact. candicans		White, flat-convex, transparent
	Myc. globiforme	tumescens	Whitish, slimy, convex, shiny
	Myc. album	–	White, convex, shiny, not transparent, large
	Micr. candidus	–	Porcelain, very convex, medium size
	Torula album	–	Porcelain, pasty, not transparent
Brown	Mycelial yeasts	–	Center flat, cream or brownish, border with fine fringe
	Bac. mesentericus	–	Flat, brownish, not transparent, sometimes folded, dull
Colorless colonies	Pseudomonas fluorescens	Does not reduce NO_3	Slightly whitish, transparent, flat, sometimes watery
	Ps. pyocianea	–	Whitish with blue pigment
	Ps. liquefaciens	–	Colorless, flat, transparent, medium size
	Ps. nonliquefaciens	–	Flat, small, transparent
	Ps. epiphytica n. sp.	–	Colorless or whitish, very convex, frequently folded with viscous slime
	Ps. liquida	–	Colorless, flat, small, transparent
	Ps. not identified	–	Flat, slimy, colorless
	Bact. liquefaciens	delucatulum healii	White, convex, transparent, shiny, somewhat cloudy, slimy, flat
	Lactobact. plantarum	–	Whitish, convex, transparent, not large
	Slimy yeasts (Torula)	–	Slimy, convex, opalescent, sometimes ropy

*Conditional terms: flat-convex—uniformly raised from edges and in center with flat surface. Small—to 2 mm in diameter. Milky—with white, somewhat cloudy, nontransparent tint. Porcelain—bright white, not transparent, with waxy gloss.

bum, P. lacticum, C. flavescens, M. candidus, mycelial yeasts.

Such results show that the representatives of the epiphytic microflora are not specifically adapted to the secretions of particular plant species, nor do they have a specific ability to inhabit only certain plant species. The data in Table 2 show that nearly all species of epiphytes are found simultaneously on a great variety of plants.

The absence of specific adaptation among epiphytic microorganisms to particular plant species was experimentally confirmed by us in the following manner: Sterilized rye seeds were bacterized with various species of epiphytic bacteria and were planted in glasses containing sterile sand or soil; the glasses were placed in a moist chamber in which the plants were

kept for 10 days. Then, an analysis was made of the green shoots, on which a pure culture of the added bacterium was usually found. A characteristic property of epiphytic microflora, in contrast to nonepiphytic, is its ability to pass from the seeds to the green parts of the plant and to live and multiply on them. Moreover, the ability of different species of epiphytes isolated from various species of plants to pass to the leaves of the same plant species confirms the fact that specific adaptation is absent among them. The epiphytic microflora can be related to a special ecological grouping, adapted to life on different parts of the growing plant—leaves, fruit, seeds, and roots. These microorganisms are distinguished by the fact that they are capable of existing under conditions of high concentration of phytoncides, reduced moisture, sunlight, and low concentrations of nutrients.

The differences in species composition of the epiphytic microflora which one actually observes on plants depend on the conditions in which the plant grew. Air humidity is of particular significance in this case. The sources from which the epiphytic microflora get on the leaves are different for different plants. For example—seeds and soil for herbaceous plants, tubers and soil for potatoes, wintering buds for trees and shrubs, etc. This also leaves a definite imprint on the species composition of the microflora of different plants.

The wide range of species of epiphytic microorganisms which we found makes it possible to state the problem of the feasibility and the means of the practical application of this microflora in a new way. Aside from its significance in the processes occurring during the ensilaging of plants or during the storage of grain, it has now been established and experimentally proved that there are cultures among epiphytic microorganisms which possess antagonistic properties with respect to fungi, including pathogenic ones (Fusarium, Botrytis, Sclerotinia). Many representatives of the epiphytic microflora secrete growth activators of the heteroauxin and vitamin type which stimulate the growth of roots. In addition, in passing from the seeds to roots, this microflora participates in plant nutrition.

SUMMARY

1. Epiphytic microflora belongs to a special ecological grouping which is adapted to life on various parts of a growing plant, such as leaves, fruits, seeds, and roots.

2. To isolate epiphytic microorganisms, it is recommended to use cabbage agar as such or one improved through addition of wort and of corn extract.

3. The species composition of the epiphytic microflora as detected in this medium is very manifold. In all, 47 species and 25 varieties have been isolated from the leaves and stems of 18 plant species.

4. In epiphytic microorganisms no specific adaptation to definite plant species has been found.

5. The differences in the species composition of the epiphytic microflora in various plant species are to be accounted for by the different conditions in which the plants are living (air humidity) as well as by the sources of this microflora.

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THE OCCURRENCE OF CELLULOSE-DECOMPOSING ACTINOMYCETES IN SOILS

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The occurrence of microorganisms in soils is closely connected with their plant formations and with the types of soils. The study concerning the coenoses of microorganisms in various soils has now been enriched by a number of investigations (Mishustin, 1954; Lazarev, 1949; Rybalkina, 1957; Sushkina, 1949, and others). Actinomycetes play an important role in the microbial coenoses of soil. Their absolute and relative numbers in soils increase from north to south. The content of actinomycetes in cultivated soils is relatively richer than in virgin ones (Mishustin, 1954, 1948; Krasil'nikov, 1958). The present work is devoted to the question of the participation of actinomycetes in the process of cellulose decomposition in various soils.

Forty soil samples from various geographic zones of the USSR were collected for the investigation. The samples differed not only in type of soil, but also in degree of cultivation. The characteristics of these samples are given in Table 1. Virgin samples were taken from horizon A1, while cultivated ones—from the arable layer.

For the isolation and quantitative counting of cellulose-decomposing actinomycetes, we used Hutchinson's medium and the method of counting the cellulose microbes of the soil recommended by Pushkinskaya (1954). Starch-ammonia medium was used for counting the total number of actinomycetes—a dilution of 1:1000. was most frequently used for counting cellulose-decomposing forms of actinomycetes, while for counting the total number of actinomycetes—a dilution of 1:1000. The inoculated Petri plates of starch-ammonia agar were placed in an incubator at 25 deg for one week. The inoculum on plates of Hutchinson's medium was kept in a moist chamber at 26 deg for two weeks.

After keeping the inocula in the incubator, the microflora that had grown up was examined, and actinomycetes were counted separately. The actinomycetes growing on cellulose were isolated and purified. The cultures were identified according to Krasil'nikov's key (1949). The ability of the actinomycetes to decompose cellulose was determined by Imshenetskii's method (1953). We gave such a description of the separate cellulose-decomposing actinomycete cultures in the preceding work, and here, only data connected with their distribution in various soils are given.

As seen from Table 2, the number of actinomycetes in northern soils is much less than in southern soils. This pertains to both the total number of actinomycetes and the group of the given microorganisms which decompose cellulose. As one moves toward the south, the number of actinomycetes in the soil increases. Chernozems, chestnut soils, and sierozems contain particularly large numbers of actinomycetes. If the numbers of actinomycetes obtained are recalculated per 1 g of humus, the pattern of their increased numbers in the soils of the southern zone can be seen even better. Such a recalculation shows that the farther south a particular soil is situated, the more actinomycetes it contains. Some reduction in numbers of actinomycetes was noted only in zheltozems. This can be explained by the fact that zheltozems have an acid reaction, and the growth of actinomycetes in them is somewhat inhibited.

A comparison of the total number of actinomycetes with the number of their cellulose-decomposing forms shows that cellulose-decomposing actinomycetes are not too frequently encountered in the soil. In southern soils, the number of cellulose-decomposing forms of actinomycetes is somewhat higher (in per cent of the total number of actinomycetes) than in northern soils. However, even here the content of actinomycete cul-

Table 1. Characteristics of the Soil Samples Studied

Place where soil sample was collected	Hydrothermal condition of the soil (water-balance coefficient)*	Type of soil	Condition of soil	Number of samples examined
Kol'skii Peninsula, Khibiny station	K > 1	Humus-ferruginous podzol	Virgin	1
			Cultivated	1
Moscow Region, Nemchinovka	K > 1	Podzol	Virgin	1
			Cultivated	5
Voronezh Region, Kamennaya Steppe; Armenia, Erevan	K = 1	Chernozem	Virgin	4
			Cultivated	9
Uzbekistan, Golodnaya Steppe	K < 1	Sierozem	Virgin	4
			Cultivated	9
Azerbaidzhan, Lenkoran'	K > 1	Zheltozem	Virgin	2
			Cultivated	3

*According to A. N. Kostyakov.

Table 2. The Occurrence of Actinomycetes in the Soils of Various Climatic Zones

Place where soil sample was collected	Type of soil	No. of soil samples	No. of actinomycetes in thousands per 1 g of dry soil		No. of actinomycetes in thousands per 1 g of humus		% actinomycete cultures decomposing cellulose
			On cellulose	On starch-ammonia medium	On cellulose	On starch-ammonia medium	
Kol'skii Peninsula, Khibiny station	Humus-ferruginous podzol	3	4.5	176	90	3520	2.5
Moscow Region, Nemchinovka	Podzol	6	33	1660	990	49800	2
Voronezh Region, Kamennaya Steppe; Armenia, Erevan	Chernozem and chestnut*	13	79	1660	2357	52950	4.4
Uzbekistan, Golodnaya Steppe	Sierozem	13	65	1550	3300	77500	4.3
Azerbaidzhan, Lenkoran'	Zheltozem	4	31	1030	620	20600	3

*Only two samples and a type transitional to chernozem.

Table 3. The Occurrence of Certain Cellulose-Decomposing Species of Actinomycetes in Various Soils

Soils	State of the soil	A. griseus	A. globispos	A. albus	A. rectus	A. roseus	A. coelicolor
Podzol	Virgin	+	+	+	+	—	—
	Cultivated	+	+	+	+	—	—
Chernozem	Virgin	+	+	+	+	—	—
	Cultivated	+	+	+	—	—	—
Chestnut	Virgin	+	+	+	+	—	—
	Cultivated	+	+	+	+	—	—
Sierozem	Virgin	+	+	+	+	—	—
	Cultivated	+	+	+	—	+	—
Zheltozem	Virgin	+	+	+	—	—	—
	Cultivated	+	+	—	—	+	+

Conditional designations: + Frequently found species; — species not found.

tures capable of decomposing cellulose does not exceed 5%.

It was interesting to analyze the distribution of individual cellulose-decomposing actinomycete species in different types of soils. We compared corresponding data for several species of actinomycetes growing on cellulose. It turned out (Table 3) that some actinomycetes, such as A. globisporus, A. griseus, and A. albus are encountered in all soils. These species have a wide areal distribution. They can frequently be found in large numbers in podzols, chernozems, chestnut soils, sierozems, and zheltozems. A. rectus was also found in many soil samples. Other species of actinomycetes—A. roseus, A. coelicolor—are encountered in mass amounts only in certain soils. For example, of the 40 soil samples examined, a culture of A. coelicolor was isolated only from zheltozem. The type of soil can evidently have a decided influence on the occurrence of actinomycetes.

The distribution of actinomycetes with different colors of aerial mycelium in different soils is interesting. As seen from Table 4, the gray and white groups of actinomycetes are often encountered in various soils and constitute a large percentage of the total number of cellulose-decomposing actinomycetes. The pigmented groups of actinomycetes appear in mass amounts only in certain soils. Among the actinomycetes producing gray colonies the majority belong to the following species: A. griseus and A. globisporus. The majority of actinomycete groups with white aerial mycelium belong to the species A. albus. A. globisporus flaveolus is encountered most frequently among actinomycetes with brown colonies. In the group of actinomycetes with pink colonies, we found two species—A. rectus and A. rectus bruneus.

The effect of soil cultivation on the actinomycete population of the soil can easily be seen from Table 4. In cultivated soils, not only does the number of actinomycetes increase, but the predominant forms also change. The properties of the soil change markedly in the process of its cultivation, which causes changes in the composition of microorganisms, including actinomycetes. However, despite the substantial changes in the actinomycete population of the soil under the influence of cultivation, it still retains the characteristics peculiar to the particular soil type.

The prolonged existence of actinomycetes in certain soils under various hydrothermal conditions not only

Table 4. The Occurrence of Various Actinomycetes in Virgin and Cultivated Soils (in thousands per 1 g of soil)

Place where soil sample was collected	Type of soil	State of the soil	Total number of actinomycete cultures studied	Colony color on Hutchinson's medium				White, %	Gray, %	Brown, %	Pink, %
				White	Gray	Brown	Pink				
Moscow Region	Podzol	Virgin	14	5	7	0	2	35.7	50.0	—	14.3
		Cultivated	48	3	38	0	7	6.2	79.2	—	14.6
Nemchinovka, Armenia	Chernozem	Virgin	23	13	4	1	5	56.4	17.4	4.2	22.0
		Cultivated	132	30	86	14	0	22.7	66.6	10.7	—
	Chestnut	Virgin	82	15	65	1	1	18.2	79.2	1.3	1.3
		Cultivated	170	39	15	96	20	22.9	8.8	56.4	12.7
	Sierozem	Virgin	69	31	13	15	10	44.9	18.8	21.8	14.5
		Cultivated	118	102	16	0	0	86.4	13.6	—	—
Azerbaidzhan, Lenkoran'	Zheltozem	Virgin	9	1	8	0	0	11.1	88.9	—	—
		Cultivated	30	0	30	0	0	100	—	—	—

Table 5. Cellulose-Decomposing Activity in Different Actinomycete Cultures

Place where soil sample was collected	Type of soil	No. of strains studied	Number of cellulose-decomposing cultures; % cellulose broken down in 1 month			
			> 40	40—20	20—10	<10
Kol'skii Peninsula, Khibiny station	Humus-ferruginous podzol	7	—	—	43	57
Moscow Region, Nemchinovka	Podzol	11	—	9	36	55
Voronezh Region, Kamennaya Steppe	Chernozem	20	—	15	35	50
Armenia, Erevan	Chestnut					
Uzbekistan, Golodnaya Steppe	Sierozem	37	5	24	41	30
Azerbaidzhan, Lenkoran'	Zheltozem	9	—	23	44	33

determines the species composition and quantitative content of actinomycetes, but also changes their ability to decompose cellulose. We tried to compare the cellulose-decomposing ability of actinomycetes isolated from various soil-climatic zones of the Soviet Union.

The experiments were set up in three repetitions in 100 ml Erlenmeyer flasks containing 50 ml of Hutchinson's medium. Ashless and starch-free filters were used. Aside from this, the filters were washed many times with hot distilled water. The inoculated medium was kept in an incubator at 26 deg. After one month, the amount of decomposed cellulose was established by Imshenetskii's method (1953).

As seen from Table 5, the actinomycete cultures which most actively decompose cellulose were found in southern soils (in sierozems, chestnut soils, and chernozems). In actinomycete cultures isolated from northern soils, the ability to decompose cellulose was reduced.

I should like to offer deep thanks to my director, Prof. E. N. Mishustin and to O. I. Pushkinskaya for valuable advice and assistance in the work.

SUMMARY

1. Cellulose-decomposing actinomycetes are widely spread in soils of different soil-climatic zones and belong to various species. The distribution area of different actinomycetes species is different. A. albus, A. griseus, A. globisporus, and A. rectus occur in great numbers in different soils. A. roseus and A. coelicolor are of a more rare occurrence, and do not occur in all soil types.

2. The number of cellulose decomposing actinomycetes increases from north to south. The podzols are poor in actinomycetes. Chernozems, chestnut soils and sierozems are abundant in cellulose-decomposing actinomycetes. In zheltozems the number of actinomycetes is small due to acidic reaction of this soil.

3. Cultivated soils are richer in actinomycetes than virgin soils. Actinomycetes isolated from southern soils exhibit a greater cellulose-decomposing activity.

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MICROBIOLOGICAL INVESTIGATION OF CARPATHIAN SULFUR DEPOSITS
I. INVESTIGATIONS OF THE NEMIROV AND LUBEN' DEPOSITS

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In recent years, more than 20 sulfur deposits have been found in the territory of the Soviet and Polish Carpathia, the reserves of which are so considerable that the Soviet Union has now moved into first place in the world with respect to known sulfur reserves.

Recently, more and more facts are appearing in the geochemical and microbiological literature which indicate the exceptionally important significance of microorganisms in processes leading to the accumulation of sulfur in sedimentary strata. On the one hand, this is indicated by the wide distribution of sulfate-reducing and thionic acid bacteria in sulfur-bearing rocks and in the waters of sulfur deposits (Shturm and Simakova, 1928; Shturm, 1937; Ivanov, 1957a), as well as by the investigation of contemporary microbiological processes occurring in sulfur deposits (Ivanov, 1957a, 1957b, 1958). On the other hand, the same thing was suggested by the study of the distribution of the sulfur isotopes S32 and S34 in various natural sulfur compounds, conducted by American geochemists on samples of sulfur-bearing rocks from large deposits on the coast of the Gulf of Mexico (Thode, Wanless, and Wallouch, 1954; Feely and Kulp, 1957).

Briefly, the content of these works and their significance in the microbiological theory of the formation of sulfur deposits can be summarized as follows. In recent years, Starkey's experiments have shown that sulfate-reducing bacteria reduce sulfates which contain the light sulfur isotope S^{32} more vigorously; due to this, reduced sulfur compounds—hydrogen sulfide and molecular sulfur, produced during the oxidation of hydrogen sulfide--have a higher percentage of light sulfur S^{32} as compared with the sulfates from which they were formed (Jones and Starkey, 1957). It was found that natural sulfur in sedimentary rocks also has a higher content of light sulfur in comparison with natural sulfates. From this, geochemists draw the conclusion concerning the microbiological character of sulfur-producing processes.

The investigation of microbiological processes in Carpathian sulfur deposits is of particular interest in connection with the fact that, to date, the genesis of these deposits has not yet been adequately studied. Therefore, the basic problem of our work is the investigation of contemporary microbiological processes in the sulfur deposits of this region for the purpose of evaluating the role of microorganisms in the formation of native sulfur and certain minerals which are connected with it genetically (sulfides, calcite, secondary gypsums, and some others), as well as to observe changes in the microbiological processes during the course of working the deposits by the open-pit method.

Industrial sulfur depositions in Carpathian deposits (Fig. 1) are correlated with chemogenic Ratinsk limestones of the lower horizon of the Upper Tortonian of the Middle Miocene (Kudrin, 1957; Sokolov, 1958). Portions of sulfur-bearing limestones are usually underlain by Upper-Tortonian gypsum anhydrides which replace limestones by phases (Kudrin, 1955), and are overlapped by a thick mass of Upper-Tortonian and Sarmatian clays (Fig. 2).

All deposits of this region are correlated with the connecting zone of the southern border of the Russian platform and the outer zone of the Carpathian depression (Fig. 1).

The hydrogeological and hydrochemical states of these deposits are characterized by the following features. Surface waters enter the water-bearing horizon of the Ratinsk limestones primarily by means of filtration through Quaternary deposits in areas of direct contact of these deposits with limestones, so that the mineral composition of the waters of this horizon forms due to the leaching of the water-containing strata themselves, that is, the limestones and the gypsums underlying them (Kudrin, 1953; Saidakovskii, 1955; Gayun, 1956; Sokolov, 1958). Within the confines of the sulfur deposits themselves, the waters of the Ratinsk water-bearing horizon are sulfate-calciferous and hydrogen sulfide-containing with a mineralization of 1.5-2.5 g/liter.

The question of the origin of the hydrogen sulfide in the waters of the Upper-Tortonian water-bearing horizon is of particular interest for both the theory of the origin of the sulfur deposits of the given region and for the practice of developing these deposits. At the present time, the majority of the geologists and hydro-

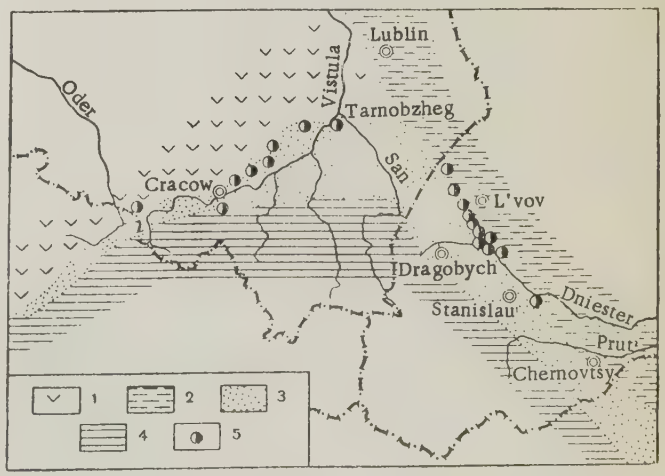


Fig. 1. Location of sulfur deposits in Soviet and Polish Carpathia in reference to the principal geostructural elements (after Sokolov, 1958). 1) Hercynian platform; 2) pre-Cambrian platform; 3) Carpathian marginal depression; 4) folded region of Eastern Carpathian mountains; 5) sulfur deposits.

chemists working in this area accept the microbiological origin of the hydrogen sulfide of these waters (Gayun, 1956; Kudrin, 1953, 1955, 1957; Gonsovskaya-Goleva, 1957); however, no special microbiological work has been done on these deposits to date. Therefore, the first task of our work was the investigation of the occurrence of sulfate-reducing bacteria in the rocks and waters of the sulfur deposits and the study of the rate of the microbiological process of sulfate reduction in subterranean waters.

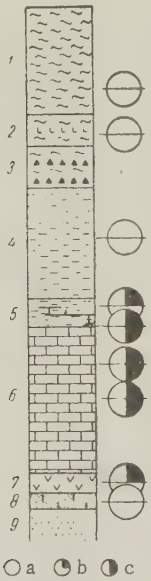
In the present communication, the results of investigations of the Nemirov and Luben' deposits are presented, the first of which is located near the Polish border, while the second is west of L'vov (Fig. 1). By now, these deposits have been explored in detail, but their exploitation has not yet begun, so that here we were able to observe the picture of microbiological processes characteristic for a deposit untouched by mining.

METHODS

The occurrence of sulfate-reducing bacteria was studied by means of plating samples of subterranean water and rocks on Tauson's agar medium (Shturm's modification) containing calcium lactate. The hydrogen sulfide content was determined by iodometric titration. The pH value was determined colorimetrically with bromthymol blue, and the oxidation–reduction potential electrometrically. All chemical analyses were made in the field immediately after collection of the water sample. To investigate the intensity of the sulfate-reducing process directly in the subterranean water, we used a method used earlier while studying the process of sulfate-reduction in lakes (Ivanov, 1956; Ivanov and Terebkova, 1959). Radioactive sulfur-labeled sodium sulfate, $Na_2S^{35}O_4$, with an activity of 40-50 μC per liter was added to samples of subterranean water placed in jars with ground glass stoppers. After this, the jars were tightly sealed with the ground glass stoppers in such a way that no air bubbles would remain under the stopper. If there were sulfate-reducing bacteria and conditions favorable for their growth in the test water, the sulfates dissolved in the water were partially reduced to hydrogen sulfide during the time of the experiment. At the end of the experiment lasting about 24 hours, all of the hydrogen sulfide was fixed with cadmium acetate (Reznikov and Mulikovskaya, 1954) and in this form, the samples were sent to the laboratory. Then, the radioactivity of the added sulfate (A_{SO_4}), the radioactivity of the hydrogen sulfide produced (A_{H_2S}), and the total amount of sulfate sulfur (S/SO_4 mg/liter) were determined. The intensity of the sulfate-reducing process was calculated according to the formula

$$v = \frac{A_{H_2S}\,[S/SO_4]\,24 \cdot 1.06}{A_{SO_4}\,t} \text{ mg/l } H_2S \text{ per hour}$$

where t – duration of experiment in hours.



○a ◔b ◑c

Fig. 2. Occurrence of sulfate-reducing bacteria along the profile of bore No. 2069 (Luben' sulfur deposit). 1) Sandy argillaceous soils; 2) ferruginous clays; 3) clay with seams of peat; 4) dense marl clays of the Sarmatian and Upper-Tortonian; 5) clay with fragments of sulfur-bearing limestone; 6) sulfur-bearing limestone of the lower horizon of the Upper-Tortonian; 7) gypsum and gypso-anhydrite; 8) and 9) sandstone and sand of the Lower-Tortonian period. a) Rock samples which gave no growth on Tauson's medium for sulfate-reducing bacteria; b) samples in which growth of sulfate-reducing bacteria was found when inoculated without dilution; c) samples in which growth was found in the first dilution.

EXPERIMENTAL RESULTS

At the Nemirov deposit, samples for microbiological work were collected from four gushing bores: Nos. 26, 37, 1 r., and 966. All these bores were drilled to the water-bearing horizon of the Upper-Tortonian limestones and were isolated by casings through the entire thickness of the cap rocks. Bore No. 26 is located at the very edge of the sulfur deposit while the three other bores are located in the central parts of the ore body, and give sulfate-calcium water typical for the given deposit, with a rather high hydrogen sulfide content—to 130 mg/liter (Table 1). The output of the bores examined differed: whereas bores No. 966 and 1 r. are situated on areas of well-filtering limestones and have a high output, bores No. 26 and 37 are low-output ones, which indicates the encumbered influx of water to the faces of these bores.

At the Luben' deposit, samples of water from "Adol'f" spring and from the high-output exploitation bore 1 k were examined. In both cases, the water was typical for Carpathian sulfur deposits, being sulfate-calcium water with a hydrogen sulfide content of 70.4 and 93.8 mg/liter.

Distinct growth of sulfate-reducing bacteria was found in media inoculated with 1 ml of water from both Luben'-Velikii samples and from bores No. 966 and No. 26 from Nemirov. The microorganisms under investigation did not grow when the inocula were diluted. Aside from this, the occurrence of sulfate-reducing bacteria was studied in core samples of rocks along the profile of bore No. 2069 of the Luben' sulfur deposit (Fig. 2). We see that the distribution of sulfate-reducing bacteria is strictly correlated with the zone of the Upper-Tortonian water-bearing horizon of hydrogen sulfide waters: They were found in three samples of sulfur-bearing limestone (samples Nos. 5,

Table 1. Characteristics of the Output and of the Chemical Composition of the Water from the Bores Examined at the Nemirov Sulfur Deposit

Bore No.	Output, liters/minute	rH_2	Dry residue, mg/liter	pH	H_2S, mg/liter	SO_4 mg/liter
26	1.5	17.0	835	7.4	12.8	419
37	0.2	—	—	7.1	133.8	540
1 r	20	12.8	—	7.0	128.9	1074
966	150	9.7	1840	6.8	130.5	978

Table 2. Intensity of Hydrogen Sulfide Production in the Subterranean Waters of the Nemirov and Luben' Sulfur Deposits

Deposit	Place where sample was collected	Duration of experiment, hours	S/SO_4, mg/liter	Activity, cpm/liter		Intensity of H_2S production, mg/liter per 24 hours
				S/SO_4	S/H_2S	
Nemirov	Bore 26	30	139	9,120,000	0	0
	Bore 37	32	180	9,100,000	4,750	0.074
	Bore 1pr	32	356	9,370,000	8,500	0.259
	Bore 966	30	326	9,520,000	7,000	0.204
	Bore 1k	32	304	10,100,000	22,700	0.543
Luben'	Adol'f spring	33	479	8,590,000	12,700	0.547

6,7), in a sample of slightly-sulfured gypsum, directly underlying limestone (sample No. 8), and in the zone of contact of sulfur-bearing limestone with covering clays (sample No. 4). All the rest of the samples, both of covering clays (samples Nos. 1, 2, 3) as well as of underlying sandstone (sample No. 9) contained absolutely no sulfate-reducing bacteria.

The investigation of the occurrence of sulfate-reducing bacteria permits us to assume that hydrogen sulfide in the subterranean waters of the sulfur deposits examined has a microbiological origin; however, the data on the occurrence of microorganisms say nothing of whether this process is occurring at the present time, and if it is occurring, at what rate. Therefore, our attention was directed primarily to the investigation of the intensity of the sulfate-reducing process directly in samples of subterranean water.

The data of these experiments are given in Table 2. We see that in nearly all of the water tested, the process of hydrogen sulfide production from sulfates occurs at a rate easily detectable by the isotopic method. The rate of this process was about the same in both of the samples from Luben'-Velikii: about 0.5 mg of H_2S per 1 liter in 24 hours. The samples from Nemirov, where the bores examined differed greatly in output and chemical composition of the water, gave a much more variegated picture. Thus, in bore No. 26, which is located on the contour of the sulfur-bearing stratum and the water from which has a rather high oxidation--reduction potential (Table 1), the process of sulfate reduction either does not go on at all, or has such an insignificant intensity that it is not detected even by means of the isotopic method. Apparently, the high value of the potential in the water sample examined prevents the growth of sulfate-reducing bacteria, although active cells of these bacteria were found in water from the bore, and vigorous hydrogen sulfide production took place on lactate-containing medium. In samples from the three other bores from which the sulfate-calcium water typical for the deposit was issuing, sulfate-reduction was occurring at a noticeable rate; moreover, this process was occurring more vigorously in water from the high-output bores Nos. 966 and 1 r than in water from the low-output bore No. 37.

SUMMARY

1. Sulfate-reducing bacteria are widely spread in the underground sulfate-calcium waters of the sulfur deposits, Nemirov and Luben-Velikii.

2. These bacteria have been detected in all sulfur-bearing rocks of the Luben-Velikii bore. In rocks covering and underlying the sulfur layer, sulfate-reducing bacteria were absent.

3. The use of labeled atoms rendered it possible to demonstrate that the processes of microbiological reduction of sulphates to hydrogen sulfide are still taking place. The rate of this process in the sulfate-calcium waters of Nemirov amounts to 0.2—0.25 mg H_2S per 1 liter a day, and in the waters of Luben-Velikii 0.54 mg H_2S per 1 liter a day.

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THE PROTEOLYTIC ACTIVITY OF SUBMERGED CULTURES OF THE ALBOMYCIN PRODUCER WITH REFERENCE TO THE FORMATION OF THE ANTIBIOTIC

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The production of antibiotic substances in the submerged cultures of the majority of producers of actinomycete origin occurs during the second period of development under conditions which differ markedly from the initial ones. It is characteristic for the second phase of development, when intensive antibiotic production is going on, that along with mycelial growth, its autolytic breakdown also occurs. In connection with this, the study of the proteolytic enzymes of actinomycetes, and especially their activity in the dynamics of the growth and development of the culture, becomes meaningful.

It has been shown in the works of a number of authors that the majority of actinomycetes have active proteases of an exogenous character (Bekhtereva et al., 1958; Surikova and Rudakova, 1958; Dion, 1955). The optimal conditions for the activity of proteolytic enzymes are different for different organisms; they have been studied most thoroughly in the streptomycin-producing actinomycete (Surikova and Rudakova, 1958; Chaloupka, 1955; McConnell, 1950). On the basis of the study of the development process of the streptomycin producer, Shaposhnikov proposed that the maximal streptomycin yield in submerged cultures of Actinomyces streptomycini is reached when proteolytic activity is somewhat retarded, as the result of which the period of equilibrium between the processes of growth and autolysis of the producer is prolonged.

Our problem includes the study of the proteolytic capacity and the accumulation of proteolytic enzymes in the culture fluid during the course of development of the albomycin producer A. subtropicus at different levels of antibiotic activity.

In the work, two strains of A. subtropicus were used which had been kindly given to us by Professor G. F. Gauze and Professor S. I. Alikhanyan. A spore suspension of two-week-old aerial mycelium from solid pea medium served as the inoculum. During the course of development of the actinomycete, the following determinations were made: 1) Antibiotic activity by the diffusion in agar method, using a standard albomycin preparation and S. aureus LA as the test organism; 2) biomass by weight of dry mycelium and 3) proteolytic activity of the culture fluid. The latter was determined by the effect of 2 ml of culture fluid on a 4% casein solution in phosphate buffer (pH 7.8-8) in 1 hour at 37 deg. After this, amine nitrogen was determined by the copper method. Proteolytic activity was expressed as mg of amine nitrogen per 100 ml of culture fluid.

The experiments were set up in synthetic medium of the following composition (%): $NaNO_3$—0.3, starch—1.5, K_2HPO_4—0.05, $MgSO_4$—0.05, NaCl—0.1, $FeSO_4$—0.05. The medium was dispensed in 100 ml amounts in flasks which were placed on a shaker at 180 rpm, and cultivation was carried out at 28 deg. Under these conditions, the growth of the organism under investigation occurred in two phases: The accumulation of antibiotic in the culture fluid began after the maximal biomass value was reached and coincided with the start of the autolytic breakdown of the mycelium (Fig. 1). An effective method for determining the mechanism of antibiotic production is the comparative analysis of biochemical changes during growth of the organism under conditions which are favorable and unfavorable for antibiotic synthesis. Conditions which are accompanied by a low level of antibiotic activity are produced by the addition of a reduced amount of nitrogen source to the medium (0.1% $NaNO_3$) (Nefelova and Zharikova, 1959).

As shown by the data in Fig. 1, the accumulation of biomass is somewhat less intensive in the medium with reduced nitrogen content, and antibiotic activity is considerably reduced. The proteolytic activity of the culture fluid reached a maximum during the period of mycelium breakdown in both cases. Thus, the increase in proteolytic capacity, occurring during the period of the second phase of development of the organism, coincides in time with intensive antibiotic production. However, the level of proteolytic activity under conditions of high antibiotic yield is considerably lower than under conditions of less antibiotic activity.

A similar analysis was done during the growth of two different A. subtropicus strains—strain 738, used in the above-mentioned experiment, and strain 614—in medium containing 0.3% $NaNO_3$. A comparison of the two strains showed that, although growth of their biomasses differed little, the production of antibiotic was considerably more intensive in strain 738 (Fig. 2). The intensity of proteolytic enzyme activity of the culture fluid from strain 738, which synthesized a larger

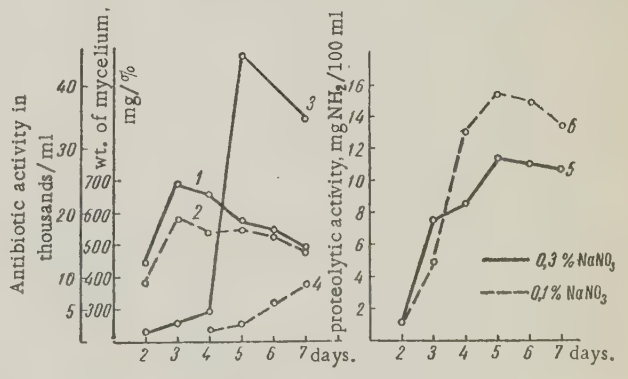

Fig. 1. Dynamics of development of A. subtropicus in synthetic medium with various $NaNO_3$ contents.

1,2) Weight of dry mycelium; 3,4) antibiotic activity; 5,6) proteolytic activity.

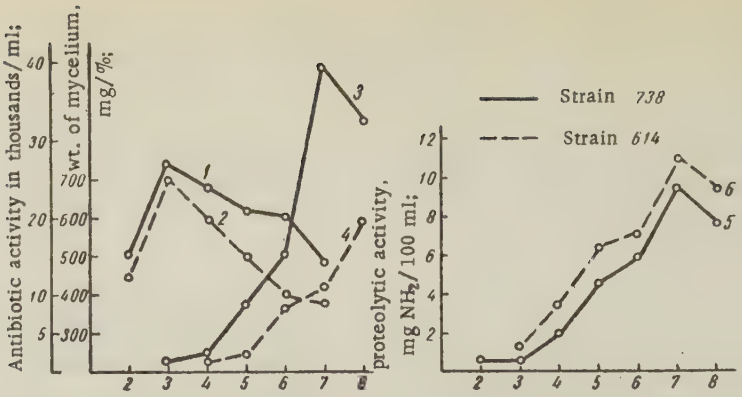

Fig. 2. Dynamics of development of two A. subtropicus strains. Key same as Fig. 1.

Table 1. The Effect of $MgSO_4$ on the Activity of Proteolytic Enzymes in the Culture Fluid During the Growth of A. subtropicus in Synthetic Medium (in mg NH_2/100 ml)

Days of growth	Content of $MgSO_4$		
	0.05% control	0.1%	0
4	1.75	1.40	2.45
5	5.25	2.45	8.40
6	5.60	4.20	10.85
7	4.2	2.55	9.10

amount of antibiotic, was somewhat lower than from the antibiotically less active strain 614. These results also agree with the microscopic picture of the mycelium of the two strains during this period of development. A larger number of disintegrating mycelial segments and fewer growing, basophilic filaments were observed in strain 614.

Thus, the low level of antibiotic biosynthesis, which depends on culture conditions or on the properties of the strain, was accompanied by increased activity of the proteolytic enzymes in the culture fluid of the actinomycete under investigation. This brought up the question of determining the reverse dependence: How will a change in proteolytic activity affect the biosynthesis of the antibiotic in growing submerged cultures

According to the data in the literature, a large number of substances including the ions of certain metals (Fe, Zn, Mg) inhibit or stimulate the activity of proteolytic enzymes at certain concentrations. After checking a number of substances, we selected Mg for the work which, in the form of $MgSO_4$, is included in the composition of the basic medium in the amount of 0.05%, which constitutes $4 \cdot 10^{-3}$ M Mg. When the $MgSO_4$ content of the medium was increased (0.1%), the activity of the proteases in the culture fluid was depressed; when $MgSO_4$ was excluded from the medium, a stimulation of proteolytic enzyme activity was observed (Table 1).

Parallel analysis of antibiotic and proteolytic activity showed that when proteolysis was retarded by increasing the amount of $MgSO_4$ in the medium, a reduction in antibiotic activity occurred as well (Fig. 3). However, the addition of an increased amount of $MgSO_4$ to the medium at the start of growth of the organism also affected its general development; some other enzyme systems were probably blocked, because growth of the biomass was retarded. Therefore, we employed a supplementary addition of Mg to the basic medium with 0.05% $MgSO_4$ on the third and fourth days of growth of the actinomycete, i.e., at the time when the maximal biomass value had already been reached and autolysis, accompanied by increased proteolytic activity, had started. In this case, the level of proteolytic enzyme activity decreased, while antibiotic activity rose (Fig. 3, III).

The omission of $MgSO_4$ from the medium, without affecting the accumulation of biomass, was accompanied by an increase in the proteolytic activity of the culture fluid. Proteolytic enzyme activity started earlier and reached a higher value than in the control. The character of antibiotic accumulation also changed simultaneously; the maximum of antibiotic activity shifted to the earlier hours of growth of the organism, but the general level decreased in comparison with control conditions. The supplemental addition of $MgSO_4$ on the third and fourth days of growth caused a reduction in proteolytic activity and an increase in antibiotic activity in this case as well. The over-all results of these experiments in the form of maximal values of antibiotic and proteolytic activities are presented in Fig. 4.

The results obtained in the work make it possible for us to propose that the autolytic process, and consequently the level of proteolytic enzyme activity as well, plays a definite role in the process of antibiotic biosynthesis by the organism under investigation. As the result of the autolysis of a certain part of the mycelium, the medium becomes enriched with the products of its decomposition which can be utilized by the mycelium growing under these altered conditions and carrying out the synthesis of antibiotic. The retardation of autolysis inhibits the accumulation of antibiotic. The stimulation of proteolytic enzyme activity causes an increase in autolysis and a decrease in mycelial growth, which also reduces antibiotic biosynthesis.

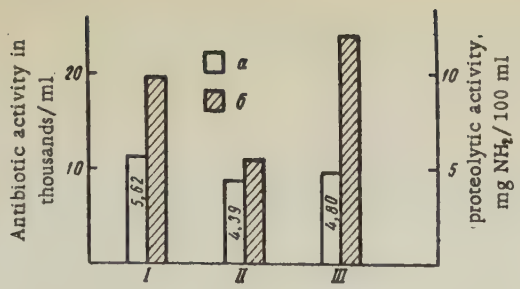

Fig. 3. Maximal values of antibiotic and proteolytic activities at different contents of $MgSO_4$ in the medium. I) 0.05% $MgSO_4$ (control); II) 0.1% $MgSO_4$; III) 0.05% $MgSO_4$ added to control medium on both the third and fourth days of growth. a) Proteolytic activity; b) antibiotic activity.

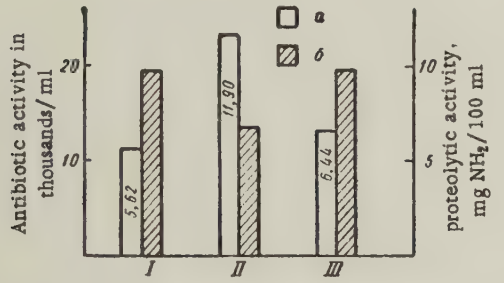

Fig. 4. Maximal values of antibiotic activity at different contents of $MgSO_4$ in the medium. A) Antibiotic activity in thousands/ml; B) proteolytic activity, mg NH_2/100 ml. I) 0.05% $MgSO_4$ (control); II) 0 $MgSO_4$; III) 0.05% $MgSO_4$ added to medium without $MgSO_4$ on both the third and fourth days of growth. a) Proteolytic activity; b) antibiotic activity.

Some delay of the proteolytic process after it has once started brings about increased activity of the growing part of the mycelium, and along with this, greater antibiotic biosynthesis as well.

In conclusion, we offer our deep gratitude to Professor V. N. Shaposhnikov for valuable advice and guidance in conducting the present work.

SUMMARY

1. In the process of growth of Actinomyces subtropicus on synthetic medium, proteolytic enzymes accumulate in the culture fluid, reaching a maximal value in the period of the second phase of development of the organism.

2. Increased biosynthesis of the antibiotic is characterized by a lesser activity of the proteolytic enzymes of the culture medium.

3. Artificial increase or decrease in the activity of the proteolytic enzymes in the culture fluid of the developing actinomycete is accompanied by a change in the level of the antibiotic activity.

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THE DESTRUCTION OF CELL WALLS OF THE BARLEY ENDOSPERM BY A CYTASE PREPARATION FROM TRICHOTHECIUM ROSEUM

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During the malting of grain, the processes connected with the action of various enzymes are of great significance. Here, a great role is played by the cytolytic enzymes which destroy cell walls and in this way facilitate the action of other enzymes.

The cell walls of the endosperm consist principally of hemicelluloses, the composition of which includes hexosans and pentosans.

The structure of the cell wall has not yet been established; however, it is considered that it consists of a more stable central lamina on which easily split high-molecular carbohydrates, polysaccharides, are situated (Luers, 1928a, b).

Brown and Morris (1890) proposed that cytase, as in the case of amylase, is secreted into the endosperm from the cyme, and showed that the destruction of the cell walls of the endosperm precedes the action of amylolytic enzymes.

Newcombe (1899) found hemicellulase which was also active in the form of an aqueous extract, in date, barley, and white lupine shoots. Gruss (1899, 1902) followed the process of cell wall destruction microhistologically. According to his observations, when the cytolytic enzyme penetrates the cell walls, it causes their swelling and acts hydrolytically. In this case, the partially dissolved walls at first become glassy and poorly visible in transmitted light, and later disintegrate and disappear completely.

Highly active cytolytic enzymes were obtained by Newcombe (1899) from Aspergillus oryzae and by Wemer (1896) from A. Wentii.

A detailed investigation of fungal cytolytic enzymes was conducted by Schellenberg (1908), who found that fungi can not dissolve pure cellulose, but act on hemicellulose.

Luers and Volkamer (1928a, b) showed that the temperature optimum of malt cytase is at 45 deg, the pH optimum is 5.0, and that heating at 60 deg for 15 minutes completely inactivates the enzyme. When acting on xylan, as much as 75% xylose is formed. The authors obtained a cytase preparation by repeated adsorption with alumina in a weakly-acid solution (pH 5.0) and elution with phosphate buffer at pH 8.3.

Cytase is produced in considerable quantities by Merulius lacrimans, which is a serious pest due to its ability to destroy wood (Basov, 1948). However, the process of cytase production by these fungi is long, since the fungi grow very slowly.

The purpose of the given investigation was to obtain cytolytic enzymes, the use of which would make it possible to utilize considerable quantities of unmalted barley and difficulty broken-down malt in beer brewing.

According to our investigations, some fungi of the Fungi Imperfecti family are distinguished by rapid growth and, on media of simple composition, produce powerful cytolytic enzymes which, in the process of mashing unmalted barley or difficultly broken-down malts, destroy the cell walls of the endosperm in a short period, facilitating the access of amylolytic enzyme to starch and of proteolytic enzymes to grain proteins and speeding up the processes of destruction of these substances.

The work was carried out with the imperfect fungus Trichothecium roseum, isolated by N. V. Pokrovskaya from the fruit of the papaya tree Carica Papaya which was obtained from China.

In order to obtain a cytolytic preparation, the fungus in the given work was grown on a medium consisting of three parts of sawdust, one part of wort solid wastes, and six parts of water acidified to pH 5.0 with lactic acid, and 0.4 parts of yeast autolyzate; it was cultured for 5-7 days at 22-25 deg. The medium was sterilized for 30 minutes at a pressure of 1 atmos prior to inoculation.

The cytolytic enzyme preparation consisted of a nutrient sawdust medium intergrown with fungal mycelium which had been dried in a stream of air at 30 deg, or the fluid pressed out of such a medium.

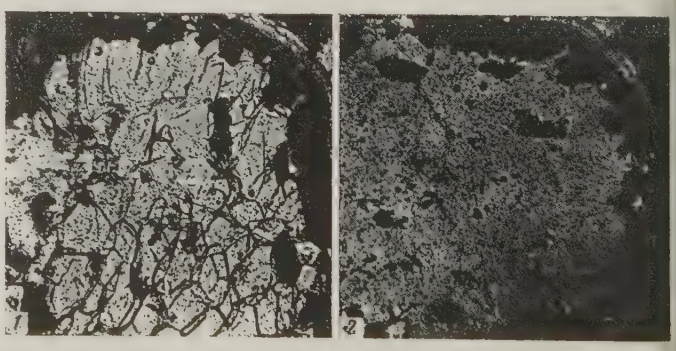
The cytolytic activity of these preparations was determined by their action on the cell walls of barley sections under the microscope.

The figure shows the effect of cytolytic enzymes of the culture fluid pressed out of a 5-day fungal culture on the cell walls of barley grains at 30 deg.

Under these conditions, the cell walls of barley endosperm underwent complete destruction in 30 minutes.

The cytolytic preparation did not contain amylases, but an insignificant amount of proteolytic enzymes was found in it.

The experiments on the saccharification of unmalted barley were carried out in the following manner: Unmalted crushed barley, in amounts of 50-60% of the total dry substances of the mash, was given a prelim-



The effect of fungal cytase on the cell walls of a section of barley grain in 30 minutes at room temperature. 1) Section of grain prior to the action of cytase; 2) same, after the action of cytase.

Table 1. Biochemical Indicators of Worts Obtained by Using 50% Unmalted Barley Treated with a 1/15 Part of the Fungal Culture Fluid (with respect to total mash)

Time of cytase action in hours	Specific gravity of worts at 20 deg	Density	RS for maltose, %	Saccharification time, minutes	Filtration time	Acidity in ml of 1 N NaOH sol. per 100 ml	Color in ml of 0.1 N iodine sol. per 100 ml
Control (2 hours)	1.057	14.039	11.5	180	5h 40'	2.40	0.61
1	1.062	15.207	12.6	120	2h 5'	2.62	1.43
2	1.062	15.207	13.5	120	2h 2'	2.74	1.77
3	1.062	15.207	13.5	45	1h 30'	2.78	1.97

Table 2. Biochemical Indicators of Worts Obtained by Using 50% Unmalted Barley Treated with Various Amounts of Fungal Culture Fluid and with a 2% Dried Culture of the Fungus after Pressing the Fluid Out of It

Amount of fungal culture fluid, ml	Specific gravity at 20 deg	Density	RS for maltose, %	Saccharification time, minutes	Filtration time, minutes	Acidity in ml of 1 N NaOH sol. per 100 ml	Color in ml of 0.1 N iodine sol. per 100 ml
Control	1.056	13.80	11.71	20	63	2.32	0.46
5	1.056	13.80	12.20	15	35	2.54	0.51
10	1.066	13.80	12.40	15	33	2.56	0.65
15	1.056	13.80	12.90	15	36	2.76	0.74
20	1.063	15.44	13.03	15	32	2.90	0.79
2% dried fungal culture after pressing out fluid	1.058	13.80	12.90	15	30	2.50	0.51

inary treatment with the preparation at 40 deg; then, the malt was added and saccharification was carried out with cooking.

The first experiments were carried out with the fluid pressed out of the fungal culture. In these experiments, the optimal time for the action of the enzyme was established and its dosage was selected. In subsequent experiments, the barley was treated with 2% dried fungal culture.

The results of all the experiments are brought together in Tables 1-4.

As seen from Table 1, the preliminary treatment of unmalted barley with the fungal culture fluid for 1-3 hours at 40 deg increased the content of reducing substances in the mash by 1.1-2% and at the same time speeded up the process of wort filtration considerably and also increased its color and acidity. The optimal yield of extractives was noted with a 2-hour cytase treatment.

The data in Table 2 are evidence of the fact that, due to the action of fungal culture fluid used in amounts of from 1/90 to 1/22 parts of the total mash, the content of reducing substances in the wort increased from 0.5 to 1.3%, filtration was speeded up noticeably, and the color and acidity of the finished worts increased.

The fungal culture fluid should be used in an amount of 1/30 part of the total mash (10 ml in the experiment) for the treatment of unmalted raw material, since with this amount, along with the increase in reducing substances in the wort and the more rapid filtration of mashes, only an insignificant increase in acidity and color of the wort was noted. Treatment of unmalted barley with 2% dried medium intergrown with fungus after the culture fluid has been pressed out of it gave the same effect with regard to the increase in reducing substances in the wort, increased filtration time, acidity, and color of the worts as when 5 ml of culture fluid was used.

Biochemical data are given in Tables 3 and 4 on wort and beer obtained by using 60% unmalted barley and 2% dried medium intergrown with fungal mycelium. The scale of the experiment in relation to factory experiments was 1:1000.

As seen from Tables 3 and 4, preliminary treatment of unmalted barley by the cytolytic preparation increased the yield of extractives in wort. In the finished experimental beer, the alcohol content was somewhat higher and the color and acidity also increased.

The observations on the viscosity of wort and the clarification of beer during fermentation and after-fermentation are very interesting. Thus, the viscosity of wort in the control was 19.2, while in the experiment, it was 14.5. The length of fermentation under identical conditions was shortened by half in the experiment as compared with the control. A difference in the clarification of the beer during after-fermentation was particularly noticeable: The experimental beer became clarified rapidly and well from the first days of fermentation, while in the control beer, this process went on more slowly and not as well. Filtration of the

Table 3. Biochemical Characteristics of Worts and Finished Beer Prepared from 60% Unmalted Barley Previously Treated with Dried Fungal Culture (Experiment 4)

	Wort							Beer				
	Amount of wort liters	Density, deg Balling	RS for maltose, %	Acidity in ml of a 0.1 N NaOH sol. per 100 ml	Color in ml of a 0.1 N iodine sol. per 100 ml	Viscosity at 20 deg	Length of fermentation, days	RS for maltose, %	Alcohol by weight, %	Acidity in ml of a 0.1 N NaOH sol. per 100 ml	Color in ml of a 0.1 N iodine sol. per 100 ml	Viscosity at 20 deg
Experimental	13.0	11.3	7.62	1.40	11.2	14.5	5	1.68	2.97	1.93	0.63	13.8
Control	12.8	11.1	7.18	1.33	Very turbid, determination difficult	19.2	7	1.71	2.79	1.81	0.50	16.8

Table 4. Composition of Nitrogenous Substances and Dextrin in Beer (Experiment 4)

	Total nitrogen in ml per 100 ml	Fractions of nitrogenous substances according to Lundin									Dextrins, %
		A			B			C			
		mg	%	Changes, %	mg	%	Changes, %	mg	%	Changes, %	
Experimental	42.4	13.42	31.6	−9.5	6.72	15.8	+4.6	22.26	52.5	+4.9	3.88
Control	41.0	16.85	41.1	0	4.62	11.2	0	19.53	47.6	0	3.85

finished experimental beer also improved considerably; it filtered rapidly, well, and with a sparkle.

In composition if nitrogenous substances (Table 4), the experimental beer compared favorably with the control in that it had 9.5% less high-molecular nitrogenous substances (fraction A), but more nitrogenous substances of average and low-molecular weight (fractions B and C).

The amount of dextrins was the same in the experiment and in the control. When kept in the cold, fewer turbid substances precipitated in the experimental beer as compared with the control.

In addition to the experiments described on the application of the cytolytic enzyme preparation for saccharifying mashes with 50-60% unmalted barley, it was also given a preliminary test in an experiment on the preparation of kvass wort from rye malt and flour. Treatment of kvass mash for three hours at 40 deg with 1/4 part (with respect to mash) of fungal culture fluid pressed out of medium intergrown with fungus before steeping, noticeably liquefied the mash, increased the content of reducing substances, increased the acidity of the wort, and reduced its viscosity.

SUMMARY

1. A technique has been developed for cultivating the fungus Trichothecium roseum with the view of accumulating cytolytic enzymes in a medium composed of sawdust with solid wastes of wort and yeast autolysate during 5-7 days at 20–25°.

2. The enzyme preparation destroys the cell walls of barley endosperm within 1-1.5 hours at 30°.

3. The use of these enzymes for the preparation of nonmalted raw material in beer mash accelerates the saccharification process and increases proteolysis of proteins by the malt enzymes.

4. In beer and kvass mashes, the enzyme preparation reduces the viscosity of wort and appreciably accelerates filtration of the mash and clarification of beer during after-fermentation.

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THE PRODUCTION OF PROTEOLYTIC ENZYME PREPARATIONS FROM PENICILLIUM CHRYSOGENUM MYCELIUM

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Interest in the production of enzyme preparations from microorganisms has increased in recent years in connection with the growth of a number of branches of industry in which enzymes are used, as well as the application of enzymes for therapeutic purposes.

At present, the enzymatic activity of various representatives of the genus Aspergillus has been the most thoroughly studied. Fungi of the genus Penicillium also have an enzyme complex which is quite wide in its spectrum. Interest in the penicillia arose in the fifties of our century in connection with the fact that a number of the species of this genus produce penicillin. Thibodeau and Macy (1942) noted the proteolytic capacity of Penisillium roqueforti. Povolotskaya and Skorobogatova (1947), while comparing the proteolytic activities of P. crustosum and P. notatum, established the greater activity of a submerged strain of P. notatum. The accumulation of proteolytic activity in a penicillium filtrate was studied by Masuda (1948). Ito (1950) studied the optimal values of acidity and stability of the enzyme preparation which he obtained from P. notatum mycelium. The dependence of the proteolytic activity of P. caseicolum on the incubation temperature was studied by Dolezalek (1956). The changes of the enzymatic activity of penicillia with age were studied by Katsnel'son (1955) and by Mattison (1956). Chost's work (1952) was devoted to a comparison of the proteolytic capacity of P. purpurogenum and P. frequentans during growth on synthetic medium. The possible practical application of the proteolytic enzymes of penicillium mycelium in the leather industry for the dehairing and softening of the raw material was first established by Yakimov and Mattison (1949). Veselov and Salmanova (1957) demonstrated the feasibility of using the proteases of dried and alcohol-treated penicillium mycelium as a beer stabilizer.

However, the direct utilization of mycelium is connected with difficulties in transportation and storage. In view of this, it would be of decided interest to obtain stable enzyme preparations. Aside from this, the production of dry enzyme preparations opens wide perspectives for their utilization by other branches of industry and medicine.

The present work was carried out for the purpose of obtaining dry, stable enzyme preparations of proteases from the mycelium of P. chrysogenum, strain "new hybrid" (beige), with the antibiotic industry as the basis. The course of the investigation was directed toward the study of the conditions for producing preparations of proteolytic enzymes and their characterization.

Successive freezing and thawing of the mycelium, as well as its autolysis were tested as the most suitable methods for extracting the enzymes from the cells. The selection of these methods was determined by the necessity for achieving rather complete extraction of the enzyme by the simplest method. The extent of extraction was determined by the accumulation of amine nitrogen in the liquid phase by Tsuverkalov's modification (1941) of the Van Slyke method, by the increase in the content of carboxyl groups—by Willstatter's method (Belozerskii and Proskuryakov, 1951), and by the amount of dry material, as well as by means of comparing the activity of the proteases and the yields by weight of the enzyme preparations. Mycelium of the fourth, fifth, and sixth stages of development, separated from the native solution on a vacuum-drum filter, was subjected to treatment. The residual moisture of the mycelium was 82-88%.

The expediency of carrying out a 24-48-hour autolysis of the mycelium at 37 deg without supplementary addition of water was established by means of experiments.

Next, a study was made of the conditions for extracting enzyme preparations from autolyzates, where ethanol, methanol, and acetone was used as precipitating agents. The addition of four volumes of ethanol or methanol, or three volumes of acetone to one volume of autolyzate was found to be the most suitable. Due to the rapid irreversible inactivation of moist preparations, they were dehydrated with ethanol and absolute ethyl ether.

The results of the experiments are presented in Table 1, which show that, using methyl alcohol precipitation, preparations were obtained having a yield (in per cent weight of dry mycelium) of 8.6-11.6%, and activity corresponding to 66-86 mg of amine nitrogen per 1 g dry mycelium. When precipitated with ethanol, the yield of preparations reached 12.0%, while activity corresponded to 47-93 mg of amine nitrogen per 1 g of dry mycelium. In the case of acetone precipitation, however, the yield fluctuated in the range of 9.6-16.1%, while the activity obtained from 1 g of dry mycelium constituted 40-105 mg of amine nitrogen. The activity per unit weight (1 mg) of the preparations separated out by means of all of the above-mentioned precipitants fluctuated within the same limits (0.600-0.800 mg of amine nitrogen). Consequently, it can be said that approximately the same results were obtained by using methanol, ethanol, or acetone.

The experiments done have made it possible to propose the following scheme for extracting proteases from mycelium: 1) Autolysis of mycelium at 37 deg for 24-48 hours without supplementary addition of water; 2) precipitation of enzymes with organic solvents; 3) drying of enzyme preparations with ethanol and absolute ethyl ether.

Table 1. The Yield and Activity of Enzyme Preparations Obtained with Different Precipitants

Preparation No.	Precipitant	Amount of autolyzate used in experiment in ml	Yield of dry preparation in g	Activity of 1 mg of preparation, mg $N-NH_2$	Yield of preparation in % of dry mycelium	Activity obtained from 1 g of dry mycelium, in mg $N-NH_2$
9	Ethanol	200	7.68	0.74	11.40	84.6
13		100	2.38	0.53	10.48	52.8
14		100	2.48	0.73	10.63	47.0
39		100	3.46	0.80	11.54	93.4
43		100	3.53	0.67	11.96	76.0
49	Methanol	100	3.12	0.74	9.0	66.7
51		100	4.03	0.74	11.6	86.4
52		100	2.99	0.80	8.6	68.4
41	Acetone	100	3.84	0.66	12.76	83.8
42		100	4.85	0.66	16.10	105.3
46		80	2.87	0.40	9.63	40.2

Table 2. Composition of Enzyme Preparations

Preparation No.	Precipitant	Moisture in % of air-dried weighed portion	In % of absolutely dry preparation					
			Ash content	Total nitrogen	Protein nitrogen	Protein (protein nitrogen 6.25)	Soluble protein	Denatured protein
9	Ethanol	9.53	34.1	9.96	3.67	23.0	12.58	10.42
13	Ethanol	10.73	26.8	9.99	4.44	27.7	13.78	13.92
41	Acetone	8.78	27.7	10.18	2.16	13.5	12.58	0.92
51	Methanol	12.38	23.5	7.42	2.68	16.75	14.47	2.28

The preparations obtained by this scheme are grayish, powdery substances. A total of 80 preparations were isolated from 28 batches of mycelium.

The second stage of the work was the study of the preparations obtained. For this purpose, moisture (drying), ash content (calcination), total nitrogen content by the Kjeldahl method, and the content of protein nitrogen by the Bernstein-Stutzer method were determined in several preparations.

Since the preparations were not completely soluble in water, the per cent protein content of the solution was determined colorimetrically using biuret reagent. The results obtained are presented in Table 2.

The data given in Table 2 are evidence of the approximately equal content of soluble protein in preparations extracted by different solvents. Inasmuch as the indicated preparations also had the same enzymatic activity, it can be considered that proteins which did not possess proteolytic activity were denatured during ethanol precipitation. In the case where methanol and acetone were used, however, a more selective precipitation of proteases probably occurred.

The activity of the preparations was tested on casein, since it is the most universal in its amino acid composition. Parallel to this, their action on gelatin, peptone, and skin powder was checked.

For the given substrates, the reaction of the medium optimal for the action of the enzymes was determined in the range of pH 5.6-9.3 for casein, 5.3-8.0 for gelatin, 5.4-7.8 for peptone, and 5.2-8.6 for skin powder (Table 3).

It was established that in the indicated range of pH values, the greatest activity is seen on casein, and the least—on skin powder. The optimal pH zone for casein was in the range of 7.3-8.2, when the duration of proteolysis was 24 hours. With longer proteolysis, two zones of maximal activity were observed: one at a weakly acid reaction of the medium, and a second at a weakly alkaline reaction.

When acting on gelatin, the greatest activity was observed at pH 6.5; another less significant maximum was noted at pH 7.6. The proteolysis of gelatin ceased after 24 hours.

The dynamics of the liquefying capacity of the preparations was studied by means of determining the viscosity of the solutions, and it was established that the disaggregation of gelatin occurs almost instantaneously. Later, only a slow drop in viscosity was observed.

The action of penicillium proteases on peptone was most actively manifested in the weakly acid and weakly alkaline zones. The activity was least at a neutral

Table 3. The Effect of pH of the Medium on the Proteolysis of Various Substrates

Casein				Gelatin			Peptone				Skin powder			
pH		Increment in mg per 1 mg of preparation, hours		pH		Increment in mg per 1 mg of preparation, hours	pH		Increment in mg per 1 mg of preparation, hours		pH		Increment in mg per 1 mg of preparation, hours	
Of buffer	Of original mixture	24	48	Of buffer	Of original mixture	24	Of buffer	Of original mixture	24	48	Of buffer	Of original mixture	24	48
4.9	5.6	0.50	0.63	4.5	5.3	0.31	4.9	5.4	0.14	0.21	4.5	5.2	0.02	0.06
5.6	6.3	0.57	0.82	4.9	5.6	0.14	5.6	5.8	0.18	0.21	5.6	5.6	0.02	0.00
6.8	7.8	0.62	0.81	5.9	5.9	0.54	6.2	6.4	0.14	0.19	6.5	6.6	0.00	0.00
7.4	7.5	0.62	0.74	6.5	6.5	0.70	7.0	6.9	0.11	0.11	7.0	7.1	0.00	0.00
8.0	8.1	0.70	0.76	7.0	7.1	0.34	7.4	7.2	0.14	0.16	7.7	7.8	0.00	0.00
8.3	8.2	0.70	0.81	7.4	7.4	0.42	8.0	7.7	0.07	0.16	8.7	8.2	0.00	0.00
9.2	8.4	0.68	0.84	8.0	7.6	0.59	8.7	7.8	0.04	0.14	9.2	8.4	0.04	0.00
10.0	9.3	0.62	0.74	8.7	7.9	0.24	–	–	–	–	9.5	8.6	0.06	0.08
–	–	–	–	9.2	8.0	0.24	–	–	–	–	–	–	–	–

reaction of the medium. An increase in the duration of the proteolysis to 120 hours was accompanied by a gradual rise in amino-group nitrogen in the filtrates.

In comparing the extent of proteolysis of the different substrates, calculated per 1 mg of preparation, the following sequence can be established: casein > gelatin > peptone > skin powder.

The stability of the enzyme preparations during storage is of great importance in their practical utilization. The preparations were stored at room temperature in flasks sealed with rubber stoppers. The stability of 15 preparations obtained by various methods was investigated selectively. The activity of the preparations was first determined after two weeks, then after a month.

It was found that enzyme preparations extracted by means of ethanol were the most stable. All of the preparations tested retained 100% of their original activity during storage for one year. The preparations obtained by acetone precipitation lost 22-37% of their original activity during storage for 10 months. The preparations extracted with methanol proved to be the least stable, retaining their full initial activity for only four months.

The methods of extracting enzymes employed in the work were not specific for proteolytic enzymes, in connection with which it was of interest to examine the multiplicity of the enzymatic activity. The quantitative determination of amylase was carried out viscosimetrically and by the dilution method (Zbarskii, Zbarskii, and Solntsev, 1954), of maltase and invertase by the polarimetric method (Belozerskii and Proskuryakov, 1951), and of catalase by the gasometric method (Belozerskii and Proskuryakov, 1954).

The presence of amylase, catalase, and invertase was established.

Taking into account the fact that, in plant and animal objects, proteolytic enzymes of the papain or cathepsin type are widely distributed, the characteristic property of which is their activation by hydrogen sulfide and other compounds, determinations were made of the effect of hydrogen sulfide on the dynamics of the autolysis of mycelium, and the activity of autolyzates and enzyme preparations extracted from hydrogen sulfide-treated autolyzates.

The absence of a positive effect makes it possible to assume that the P. chrysogenum enzymes which we have studied do not belong to the papains.

SUMMARY

1. The possibility of obtaining a preparation of proteases from Penicillium chrysogenum mycelium which is stable upon storage has been established.

2. The preparation can be obtained by precipitating the mycelial autolyzate with methanol, ethanol, or acetone. The best yields and greatest stability were observed when ethanol was used as precipitant.

3. A comparative study of the proteolytic activity of the preparation on different substrates showed that the greatest activity is noted on casein, then on gelatin, peptone, and skin powder.

4. The enzyme preparation obtained probably contains trypsin-like protease.

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MICROBIOLOGICAL MACERATION OF EUCOMMIA LEAVES
I. AGENTS OF "FERMENTATION" OF EUCOMMIA LEAVES

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The leaves of the guttapercha-bearing Eucommia orm the raw material for the industrial obtention of guttapercha.

The first stage of production is "fermentation" of he leaves in heaps by the microflora present on the eaves. After this, the guttapercha is separated mehanically from the leaves which have become macrated during "fermentation". The microbiology of his "fermentation" has not been studied by anyone so ar. We only know that as a result of fermentation the eaf tissues are softened, which facilitates the extracion of the guttapercha, and there is some reduction n the amount of resins. This reduction is desirable, ince a too resinous guttapercha is unsuitable for techical purposes.

In a first communication [1959] we gave the reults of our researches on the optimum conditions or "fermentation".

This paper is devoted to a study of the microflora esponsible for the "fermentation".

Microflora of Leaves Undergoing Fermentation with Air Available.

The study of the microflora of Eucommia leaves ndergoing maceration began with a microbiological nalysis of the active complex of microorganisms which developed on damp Eucommia leaves undergong spontaneous fermentation in flasks which admitted air. After 2 weeks of fermentation, one g of wet leaves was ground up with a glass rod in 10 ml of sterile water, and then the suspension was shaken for 10-15 min.

A series of tenfold dilutions was then made. Each dilution was used to inoculate different media for revealing the different physiological groups of microbes.

The presence of microorganisms decomposing cellulose in aerobic conditions was determined from the development on filter paper partially submerged in Hutchinson's mineral nutrient. Anaerobic cellulose-decomposing microorganisms were revealed on filter paper soaked with a medium containing 0.2% $(NH_4)_2$—HPO_4 and the other usual mineral constituents, with 0.3% peptone added.

Aerobic pectin-decomposing bacteria were observed on agar plates containing 0.4% water-soluble pectin as a carbon source, this pectin being obtained from the white of oranges by Proskuryakov's method [1939]. As an amino acid source, 0.04% peptone was added to the medium. Anaerobic pectin bacteria were detected in liquid medium of the same composition, contained in test tubes and sealed with a layer of paraffin after inoculation. The development of pectin bacteria in this medium was indicated by its turbidity and the accumulation of gas under the paraffin seal.

Putrefying aerobic bacteria were observed on MPA in Petri dishes, and anaerobes in a column of the same medium in test tubes.

Aerobic pectin-utilizing microorganisms were found in numbers $1 \cdot 10^6$—$1 \cdot 10^8$ per one g of wet weight of leaves. Pectin-decomposing anaerobes developed in inoculations from the 8th-10th dilutions, i.e., their number reached $1 \cdot 10^{10}$ per g of wet leaf weight.

The question arose of whether the pectin bacteria were facultative anaerobes, i.e., were those found in aerobic and anaerobic conditions of the same type. For a solution of this question the aerobic bacteria grown on the plates were seeded in anaerobic conditions, and the purified anaerobic cultures forming colonies in the column of pectin agar were seeded in aerobic conditions on to plates with pectin medium. There was no growth in either case, Hence, we can conclude that the aerobic and anaerobic pectin bacteria are two different groups of microorganisms.

Aerobic cellulose-decomposing bacteria were not found in the cultures. On filter paper partially submerged in Hutchinson's medium, there grew fungi which produced a black pigment. They were present in fairly high numbers—they were found up to the 5th-6th dilutions.

Cellulose-decomposing anaerobic bacteria were not found at all, or the number found was only $1 \cdot 10^2$ per one g of leaf.

The content of putrefying anaerobes was $1 \cdot 10^6$ cells per one g of wet leaf.

Molds developed abundantly on the leaves. The whitish mycelium on the leaves could easily be seen with the naked eye. To isolate the fungal microflora typical of Eucommia leaves we made inoculations onto agar medium in which the only food source consisted of finely ground leaves in amount 5%. On this medium there was a rich growth of molds of the genera Aspergillus, Penicillium, and Chaetomium.

Thus, in the complex of microorganisms macerating Eucommia leaves, there predominate pectin-decomposing aerobic and anaerobic bacteria, putrefying bacteria, and molds. The presence of anaerobes in the fermentation of leaves, when air is available, is not surprising in view of the fact that, as the maceration of the leaves progresses, they fuse into solid clumps inside which anaerobic sonditions are created.

Microflora of Leaves Undergoing Fermentation Without Access to Air.

In the microbiological analysis of leaves undergoing maceration in anaerobic conditions, we also found hemicellulose-utilizing bacteria in addition to the physiological groups determined above. As hemicellulose we used the water-insoluble part of the orange white. To promote the development of cellulose bac-

Table 1. Count of Microorganisms Macerating Eucommia Leaves in Anaerobic Conditions

Aerobes			Anaerobes				
						cellulose-decomposing	
Putrefying	Pectin	hemicellulose-utilizing	Putrefying	Pectin	hemicellulose-utilizing	on filter paper	on filter paper pectin
$2 \cdot 10^7$	10^7	10^8	10^7	10^7	10^7	10^3	10^6

teria we added pectin to the medium in addition to the filter paper. We determined the number of microorganisms per one ml of a suspension obtained by grinding one g of fermented leaf in 3 ml of culture fluid. The results of the analysis are given in Table 1.

As the results in Table 1 show, the decomposition of cellulose when pectin was present was observed in inoculations at higher dilutions. On microscopic examination of the decomposing cellulose fibers we found in all cases slender rods with spherical plectridial spores, which, in all probability, were typical cellulose-decomposing bacteria.

The observations indicate that in the anaerobic maceration of Eucommia leaves there participate not only pectin-fermenting agents, but also anaerobic cellulose-decomposers.

Maceration of Eucommia Leaves by Individual Cultures of Microorganisms.

In order to determine which microorganisms are the main agents of maceration of Eucommia leaves, we inoculated sterile leaves with pure and enriched cultures of the assumed macerating agents.

Active enriched cultures of aerobic yellow and orange cellulose-decomposing bacteria, isolated from soil, did not produce any maceration of the leaves during a period of 47 days. More than ten enriched cultures were tested. On the basis of these observations we can conclude that aerobic cellulose-decomposers do not macerate the leaves.

From the fermented leaves we isolated 7 pure cultures of aerobic bacteria which grew on agar medium containing peptone as the only source of carbon. These bacteria, however, neither in a mixture nor separately, macerated sterile Eucommia leaves in aerobic conditions, or did so very weakly. Thus, aerobic pectin-decomposing bacteria are not the main agents of maceration.

From leaves macerated in anaerobic conditions we isolated 6 pure cultures of anaerobic pectin-fermenting bacteria. These bacteria did not macerate sterile leaves in anaerobic conditions either at pH 5.5 or at pH 7 or 8. We also tested unpurified cultures of pectin bacteria containing 2–3 aerobic associates in each. In this case also, not one out of 10 enriched cultures, nor a mixture of them all together, caused maceration. Hence, anaerobic maceration is not pectin fermentation. In a similar manner we tested active enriched cultures of anaerobic cellulose-decomposers of varied origin, containing several associated organisms, including pectin-decomposing bacteria. These cultures again, however, did not macerate sterilized leaves and did not accelerate the slow fermentation of leaves by the endemic microorganisms.

At the same time, the high content of pectin- and cellulose-decomposing bacteria in leaves undergoing maceration indicates that they participate in the maceration process.

From leaves fermented in aerobic conditions we isolated 4 pure cultures of molds: Two belonging to the genus Aspergillus, one Penicillium, and one Chaetomium. When these fungi were inoculated on to sterile leaves we found that 3 of the fungi did not cause maceration, but one, belonging to the genus Aspergillus, macerated the leaves well. With repeated subculturing the maceration was finished within 6–7 days. This fungus did not affect gutta but in 2 months destroyed 30% of the resins. It is obvious that the agents of maceration in aerobic conditions are certain molds. This question deserves special study.

All our observations indicate that maceration of Eucommia leaves is effected not by a single physiological group of microorganisms, but by a complex of them.

Complex of Microorganisms "Fermenting" Eucommia Leaves

Attempts to find the principal agent of leaf "fermentation" or to obtain a simple combination of cultures effecting this process met with no success. Maceration was carried out only by complexes of microorganisms developing spontaneously and being greatly stimulated in passages on the leaves. Even after several passages of these complexes on synthetic medium containing water-insoluble carbohydrate (hemicellulose), microbiological analysis revealed large numbers (in the 6th-8th dilutions) of aerobic bacteria growing on pectin and peptone (the same forms); of anaerobic pectin-decomposing microorganisms, the usual clostridial forms; of anaerobic cellulose-decomposing forms, typical Plectridia; of peptone-decomposing anaerobes and hemicellulose-decomposing anaerobes.

The microflora decomposing gutta and resins will be dealt with in our next paper.

SUMMARY

1. Microbiological analysis of the microbial complex causing spontaneous "fermentation" (maceration) of Eucommia leaves has revealed molds of the genera Aspergillus, Penicillium, and Chaetomium, aerobic polyphagous bacteria utilizing peptone and pectin, anaerobic pectin bacteria, anaerobic cellulose-decomposing bacteria, putrefying anaerobes, and anaerobic

ıemicellulose-utilizing bacteria. This complex mac-
ɜrates the leaves in aerobic and anaerobic conditions.

2. Pure or enriched cultures of bacteria decomposing
:ellulose and pectin, utilizing hemicellulose and pep-
one in aerobic and anaerobic conditions, do not sep-
ırately cause maceration of the leaves either in aerobic
ɔr anaerobic conditions.

3. Among the molds there occur forms (Aspergillus)
which in pure culture macerate Eucommia leaves.

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A MICROSCOPIC STUDY OF PAPER PIGMENTED BY THE FUNGUS GYMNOASCUS SETOSUS

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A substantial amount of research material on the microbiological processes occurring in paper, books and documents is now available. However, information on the microscopy of paper damaged by microbes is extremely limited.

The well-known monographs of Thaysen and Bunker (1927), Siu (1951), and Imshenetskii (1953) have summarized the numerous investigations in the field of microbiology of cellulose materials for the whole preceding period, but little is said about the destruction of paper. In original works on this subject (Beckwith, Swanson and Jiams, 1940; Nyuksha, 1953, 1956, 1957), it was noted that fungal damage to paper in Libraries and archives is accompanied by brittleness, increase of acidity, and the formation of colored products of red, yellow, grey, black, brownish, and other hues. The reduction in the strength of materials due to microbiological damage is directly related to the number of fibers damaged. Fibers treated chemically and lacking cuticle are destroyed more rapidly; fungi easily penetrate into mechanically damaged fibers. The more amorphous the fiber, the more rapidly it undergoes destruction.

Pigment spots are formed by fungi on paper when the growth of the vegetative parts of the mycelium is poor. When they grow in the form of dense colonies with abundant sporulation this effect is rarely found. Pigmentation of fibers at the site of fungal colonies is very much deeper pegmentation obtained in staining with solutions. The brownish-yellow pigmentation, which appears sooner or latter in cases of fungal damage to paper, is associated with the appearance of humic substances.

MATERIAL AND METHODS

One of the most widespread and injurious fungi is Gymnoascus setosus, which rapidly destroys paper and pigments it with a raspberry-red color.

The aerial part of the G. setosus colony during sporulation consists of grey, flocculent, loosely attached formations situated on the surface of books or at the site of folds. On examining the colony under the microscope, one can clearly see the dark, pigmented, anterlike hyphae surrounding the asci (Fig. 1). However, this part of the mycelium is usually absent, and the only obvious traces of the fungus living on the paper are red patches, from which we can establish the site of the colony. The outlines of the pigment patches follow the shape of the colony. Living mycelium can almost always be isolated from inside a fresh patch. The fungus is not usually found outside the limits of the patch. In very damp conditions, the coloring matter may leak out beyond the limits of the colony and hence, mycelium is not always present in the peripheral part of the patch. The nature of pigment formation depends on the kind of paper: Very clear, brightly pigmented patches occur on chalk-loaded paper; on other types of paper their color is duller. It has been shown that pigment production is promoted by a neutral or slightly alkaline original medium. In most cases, the greatest pigmentation is obtained when the pH is lowered to 5.4-5.1. G. setosus pigment on paper fluoresces in ultraviolet light of wavelength 366 μ, thus distinguishing it from the red pigments of fungi of the genera Pencillium and Aspergillus.

For obtaining colored specimens in experimental conditions we used circles of various kinds of paper with fiber and sizing of known composition. The circles were placed in Petri dishes and moistened with a medium suitable for the production of pigment by this fungus. The medium consisted of 1% saccharose, 0.2% peptone, and 0.02% ammonium molybdate in distilled water. After inoculation, the dishes were kept in exsiccators at relative humidity close to 100%, so that the specimens would not dry up. After the elapse of 2 months the damaged paper was studied.

In the microscopy of paper damaged by fungus, it is the usual practice to note the difference in color between the fibers and fungal mycelium. In the case in question, the hypae of G. setosus have a fairly distinct natural color. The difficulty lies in the fact that the pigment is soluble in water, and when the hyphae are mounted they become appreciably paler. To avoid this we used a method of mounting in immersion oil. The damaged pigmented paper on a slide was teased apart with dissecting needles into separate fibers, which were dried in air, so that no water bubbles would form in the specimen, but to avoid deformation of the mycelium we did not overdry them. The fibers were then spread out in a drop of immersion oil. The mounts were very clear and kept for a long time.

EXPERIMENTAL

Microscopic examination of the damaged paper showed that in the first stages of development the fungal mycelium is colorless. Sometimes, pigment production does not accompany later stages of the destruction of the fiber. In our experiments, red pigment soon appeared in the hyphae and the color gradually deepened. The pigmented protoplasm acquired granularity. In this case, the mycelium remained very slender: The diameter of the hyphae did not exceed 1 μ, and hyphae 0.5 μ in diameter were usually encountered. The hyphae wove round the surface of the paper fibers and penetrated in different directions (Figs. 2, 3, 4). The position of the hyphae and a definite orientation: They were usually located along cracks, torn edges, and along the grain of the fiber (Fig. 2). Sometimes, however, the fibers were penetrated almost perpendicular to their axis. Some of the hyphae

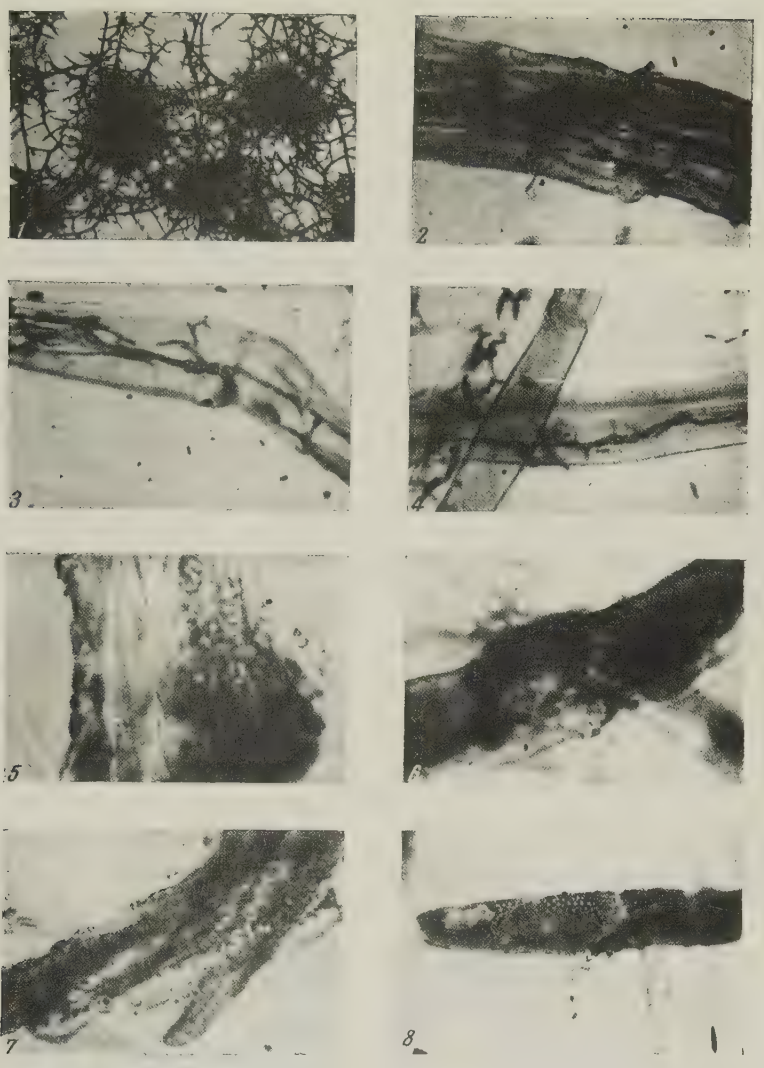

Fig. 1. Fruiting bodies of Gymnoascus setosus. Mag. 120×.
Figs. 2, 3, and 4. Paper fibers with pigmented mycelium of fungus. Mag. 600×.
Figs. 5 and 6. Paper fibers severely damaged by fungus. Mag. 1400×.
Fig. 7. Granularity of fiber due to fungal attack. Mag. 600×.
Fig. 8. Pigmented fine fiber of paper. Mag. 600×.

wound round the outside of the fiber and in places formed a sort of network on the surface (Fig. 4). Some hyphae, usually the thicker ones, passed between the fibers. In cases where the outer layers of the fiber were damaged the fungus formed a mat (Fig. 5). It gradually affected the deeper layers and penetrated between the fibrils where it formed a very dense net-like tangle. The fibers became brightly colored and lost their structure (Fig. 6). As pigmentation increased the fungal hyphae died off and their breakdown products, fragments of the colored cell contents, remained inside the damaged parts of the fibers. The progress of this process led to severe damage and destruction of the fibers, which acquired a distinct granular structure (Fig. 7). Fine fibers were deeply pigmented and suffered most damage (Fig. 8); coarse fibers, especially in the first stages of attack, were usually unaffected. On some of the fibers the mycelium was not apparent, but they acquired an entire raspberry-red color, and only the lumen and cracks had a brighter tinge.

Treatment with chlor-zinc-iodine and other methods were ineffective in revealing the different nature of the fibers if they had been subjected to the same chemical treatment. However, when the preparations were examined in the fluorescence microscope, it was found that the original filter paper treated with acridine showed the same differentiation in the color of the fibers as the fungus-damaged paper in the ordinary microscope. Fine fibers, considerably damaged by the fungus, had a golden-yellow fluorescence. Larger fibers had a yellowish tinge; they were less susceptible to fungal attack and were effected mainly at damaged parts. Lastly, coarse fibers of distinct structure, which were usually undamaged, had a lettuce-green color.

It was found that the pigmentation of the fibers by the fungus could be reproduced by staining the preparations with the usual stains used in microscopy. In our work two stains were used: a 1% solution of basic fuchsin in 70% enthyl alcohol and a 0.5% aqueous solution of methylene blue.

An aqueous suspension of fibers from paper was prepared, and a few drops of the suspension were mounted on a slide. The preparation was dried until all the water was removed, and was lightly fixed in a flame. Staining was first done with basic fuchsin for 5 min, and then the stain was washed off with water. The whole preparation usually took on an even stain. It was then treated in the same way with methylene blue for 1.5 min and washed with water. The stained fibers were dried to the air-dry state and examined in immersion oil under a cover glass.

A study of the preparations under the microscope and of photographic prints showed that this staining made it possible to distinquish fibers by their ability to take up fungal pigment and partly by their composition. Firstly, lignified fibers stained red. Basic fuchsin, being a stain with three amino groups, reacts well with lignin and gives it a fast color (Fig. 10). The red color was also taken on by fine, filmlike fibers, often devoid of structure (Fig. 11). Untreated wood, resin particles, and filler also became red. Cellulose was stained blue and the purer it was, the bluer it became. Some of the fibers took on an intermediate violet coloration.

It should be noted that with a similar method of staining, hydrocellulose prepared by the laboratory method becomes entirely blue, and oxycellulose red.

In preparations of fibers on which the fungus had grown for a long period the entire staining with fuchsin occured on the same part of the fiber as in preparations of the original paper. However, the general staining was much deeper due to the great quantity of fungal hyphae which took up the fuchsin, or were naturally pigmented.

DISCUSSION

The pigmentation of paper fibers by the fungus G. setosus can be attributed to two causes. Firstly, there

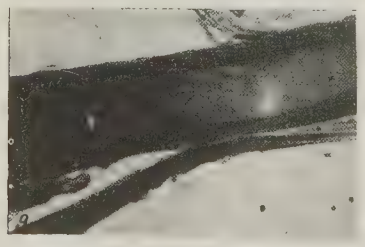

Fig. 9. Wood fiber, entirely pigmented by fungus. Mag. 600×.
Fig. 10. Wood pulp, stained with fuchsin and methylene blue. Mag. 120×.
Fig. 11. Staining of fine fibers of paper with fuchsin and methylene blue. Mag. 120×.

s a true staining of some fibers due to the fact that ignin, hemicelluloses, oxycellulose, and sizing substances take up the pigment excreted by the fungus. This takes place without visible participation of the ungus in the destruction of the given fibers. However, s presence is of undoubted significance since even rolonged keeping of paper in a concentrated solution f pigment alters its color little. In a similar way olored fibers occur in great numbers in papers containing wood pulp. Since the fungus intensifies its xcretion of coloring matter in a moderately acid nedium, approximating to a neutral or even alkaline eaction, especially good pigmentation is obtained on halk-loaded papers. On newspaper the fungus develops oorly and gives practically no pigment.

Secondly, some of the paper fibers are ensheathed n a fine network of fungal hyphae, which is sometimes o thick that the fiber, especially under low power, ppears entirely pigmented, but after removal of the nycelium it is found to be colorless. In places where ne fiber is damaged by the fungus there forms a dense nat of mycelium, which also gives the impression of igmentation. This is observed in those kinds of paper vhich contain cotton and flax fibers or purified wood ellulose.

SUMMARY

1. The raspberry-red patches formed on paper by ne fungus Gymnoascus setosus are not a uniform phenomenon. They are due, on one hand, to staining of lignin, oxycellulose, cellulose decomposition products, and sizing substances by the fungal pigment and, on the other, to the presence of a large amount of pigmented mycelium in the paper. Purified cellulose does not take up the pigment.

2. The fungal pigment behaves like a basic dye. By using solutions of basic fuchsin and methylene blue it is possible to reproduce the fungal staining pattern on paper.

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BACTERIA OF THE MAMMOCOCCUS GROUP AS ONE OF THE CAUSES OF BITTER TASTE IN CHEESE

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Bacteria of the Mammococcus group, according to published information, cause strong bitterness in cheese and give it a smearing consistency. This has been confirmed by the studies of Vereschagina et al. [1932], Bogdanov [1936], Konopleva [1955], Lane [1934], and Dean [1951].

We know that mammococci possess considerable proteolytic activity and in an acid medium they can decompose protein and give rise to bitter products.

The aim of the present work was to determine how different doses of pure cultures of Mammococcus, introduced into pasteurized milk for cheese production, affected the ripening of the cheese and its quality. Fifteen experimental batches of Dutch cheese were prepared.

In carrying out the experiments we tried to obtain different degrees of bitterness in the cheese and to determine the chemical and microbiological characteristics of the experimental cheeses.

The chemical composition of the experimental and control cheeses was determined by conventional and standard methods of investigation. For the quantitative determination of amino acids in the cheese, after their chromatographic separation on paper, we used Bode's method [1955]. The microbiological analysis was performed by standard methods.

RESULTS OF INVESTIGATION

It is generally known that the introduction of a pure Mammococcus culture into milk for cheese production results in cheese of bitter taste. In our investigations, made in three replicates, the introduction of equal doses of starter (0.4%) and Mammococcus culture (0.4%) into the pasteurized milk gave a slightly bitter cheese. The introduction of 0.5% starter and 0.5% pure Mammococcus culture (three replicates) into the pasteurized milk gave the same results—cheese with only slight bitterness.

The results obtained in a study of the ripening of the experimental and control cheeses showed that the course of the microbiological process and the concomitant changes in acidity and lactic acid content of the cheeses were typical of Dutch cheese. Changes in acidity and lactic acid content of the experimental cheeses during ripening were slight, which was due to the poor growth of the Mammococcus and the reduction in its proteolytic acitivity owing to the intense lactic acid fermentation.

Scheme of Experiments

Variants	Introduced into pasteurized milk, %	
	Starter	Mammococcus
I	0.4	0.4
II	0.5	0.5
III	0.5	2.0
IV	—	2.0
V (control)	0.5	—

The mean pH, equal to 5.22, found both in fresh cheese and in cheese 30, 60, and 90 days old, was much lower than the optimal pH, equal to 6.0, required for manifestation of the proteolytic activity of Mammococcus.

The chemical analysis of protein breakdown products consisted in the determination of the different forms of nitrogen (total soluble, protein, nonprotein, amino, ammonia, etc.).

It was found that the simultaneous introduction of pure Mammococcus culture and starter somewhat accelerated proteolysis, and altered the direction of this process towards increase in the quantity of primary protein decomposition products—peptones and free amino acids.

At the age of three months (when the control Dutch cheese was fully ripe, according to organoleptic indices) the content of soluble forms of nitrogen (relative to total nitrogen) in the experimental cheese was 1.1-1.3 times higher than the corresponding results for the control Dutch cheese.

Judged from the quantity of peptones, the process of protein breakdown progressed more rapidly towards the accumulation of primary breakdown products in the experimental cheese than in the control; this last feature is in accord with the view that bitterness in cheese is due largely to the accumulation of primary protein breakdown products—peptones.

However, the results obtained in the studies of the chemical composition of the experimental cheeses indicated that in cheese containing Mammococcus the processes went more rapidly, both towards accumulation of peptones and accumulation of amino acids. Hence, the addition of pure Mammococcus cultures to milk causes some deviation from the normal process of protein breakdown. The bitterness in the experimental cheese was probably due not only to the accumulation of peptones, but to bitter amino acids too.

By investigating the amino acid composition of control Dutch cheese and experimental cheeses containing Mammococcus during ripening, we tried to establish the relation between the quality of the cheese and the changes in its amino acid composition.

As a result of special investigations on ripe experimental and control Dutch cheeses we found 18 amino acids: aspartic and glutamic, arginine, lysine, glycine, serine, threonine, cystine, alanine, tyrosine,

minobutyric acid, valine, methionine, phenylalanine, roline, tryptophan, leucine, and isoleucine.

The total content of water-soluble amino acids in xperimental and control Dutch cheeses is given in ie table.

The obtained results showed that an accumulation f amino acids was observed both in the experimental nd control cheeses. The stronger taste corresponded o the greater quantity of amino acids in the ripening heese. The accumulation of amino acids in the cheeses as accompanied by their breakdown. Products of mino acid breakdown are responsible for the specific taste and aroma of cheese.

By preparing cheeses from the same milk we obained unripe cheeses with similar amino acids composition. However, at the age of three months the xperimental cheese contained 1.1 times more free mino acids than the control cheese. It is possible iat in this case the Mammococci, acting on proteins, onverts it into a more accessible form for the typical ictic acid bacteria, which perform the subsequent ipening process, and thus promote the more rapid reakdown of protein to amino acids.

As the cheeses ripened their content of such amino cids as the diamino acids—aspartic, glutamic+threoine and leucine+isoleucine, considerably increased.

The amount of the amino acids alanine, tyrosine -α-aminobutyric acid, glycocoll serine and valine methionine, increased until the cheese was two ionths old, and then a decrease was noted at the age f three months, while at the age of five months their mount had increased again. Both in the experimental nd control cheeses the content of tryptophan and phenlalanine also increased up to the age of two months, nd then their amount gradually decreased right up to ie age of five months.

Proline was clearly visible on chromatograms of ve-months old specimens of cheese. It is quite posible that this amino acid was present also in the young heeses, but it is difficult to detect owing to its yellow color when the chromatogram is developed with a ninhydrin solution.

In experimental cheeses, in contrast to the controls, proline and arginine predominated in quantity. The tryptophan content of the experimental cheeses was a little higher than that of the controls.

Thus, the change in the microfloral composition of ripening cheese (addition of pure Mammococcus culture to the milk) accelerated proteolysis. However, the increase in the total amount of amino acids in the experimental cheeses as compared with the controls led to a deterioration in the taste and aroma of the cheese. The accumulation of bitter-tasting amino acids—arginine, tryptophan, lysine, aspartic acid, methionine, valine, phenylalanine, leucine and isoleucine was the cause of the bitterness.

Of the organic acids participating in the production of taste and aroma we studied the changes in the accumulation of lactic acid. Since the nature of the variation of the lactic acid content of the experimental and control cheeses was approximately the same it is quite probable that lactic acid per se is not responsible for bitterness in cheeses, although it may indirectly affect the change in the protein content of the cheese, and thus under certain conditions promote bitterness.

This hypothesis is based on the fact that bitterness in cheese is often accompanied by a sour taste.

We know also that the specific taste and aroma of cheese are due not only to the accumulation of individual amino acids, but also to the conversion of the latter into simpler substances.

Such taste-forming and aromatic substances as ketones, alcohols, diacetyl, and acetone accumulated more slowly in the experimental cheese than in the control. It may be surmised that the addition of a Mammococcus culture to the milk during cheese production results in a much slower breakdown of amino acids as compared with their production. This lag leads in turn to an alteration of the quantitative relationship of the taste-forming substances, thus produc-

Changes in Amino Acid Composition in Ripening Dutch Cheese

Amino acids	Cheese							
	Experimental				Control			
	Age in days							
	30	60	90	150	30	60	90	150
Diamino acids	1.058	2.904	3.729	4.065	1.014	2.974	3.297	4.158
Aspartic acid	0.224	0.696	0.950	1.057	0.147	0.687	0.771	1.077
Glycocoll + serine	0.379	1.100	0.846	1.280	0.514	0.947	0.716	0.932
Glutamic + threonine	0.447	1.584	1.710	2.270	0.542	1.540	1.640	2.012
Alanine	0.074	0.296	0.214	0.250	0.025	0.265	0.201	0.210
Tyrosine + α-aminobutyric acid	0.461	0.678	0.311	0.795	0.401	0.888	0.370	0.425
Valine + methionine	0.338	0.602	0.634	0.805	0.337	0.658	0.386	0.665
Phenylalanine	0.207	0.917	0.419	0.359	0.157	0.632	0.352	0.440
Leucine + isoleucine	0.562	1.039	1.427	1.445	0.568	1.420	1.276	1.328
Tryptophan	0.058	0.072	0.059	0.056	0.054	0.068	0.053	0.054

Note. The data given are the averaged values from three batches of control and three batches of experimental cheeses.

ing a deterioration in the taste in general, but this is not the direct cause of bitterness in the cheese.

On analyzing the obtained data on the composition and amount of microflora in the cheeses we concluded that the dominant microflora of ripening cheeses consisted of lactic acid bacteria. In all the experimental cheeses the numbers of mammococci diminished with the age of the cheese. The reduction in the numbers of mammococci in the experimental cheese as it ripened and the weakening of its proteolytic activity were due to the active lactic acid fermentation, which in turn was due to the employment of an active starter containing all the bacteria necessary for ripening.

According to published results, the growth of Mammococcus and its proteolytic activity are inversely related to the activity of lactic acid fermentation. Hence, despite the introduction of a large number of mammococci into the pasteurized milk, we obtained in every case cheese with only slight bitterness, owing to the employment of a good active starter.

The main reason for the slight bitterness caused by the introduction of 0.4% Mammococcus culture and 0.4% starter was the simultaneous increase in primary protein breakdown products—peptones and bitter-tasting amino acids. Hence, in a study of the ripening process of experimental cheeses obtained in variants II and III of the experiment, the chemical investigations mainly involved a determination of the amount of peptones and amino acids.

Besides this, the organoleptic and microbiological indices of the cheeses were investigated. Changes in the chemical composition of the cheeses and the conditions of the medium were regulated by the usualy standard methods. In variant III of the experiment on Dutch cheese production we introduced into the pasteurized milk 0.5% of starter and four times as much, i.e., 2%, pure Mammococcus culture, so that we could find out to what extent the degree of bitterness of the cheese increased, and how far the rate of protein breakdown and accumulation of bitter products changed. By introducing a much greater number of mammococci into the milk we strove for an early suppression of the activity of the starter microflora, and thus to create congenial conditions for the growth of Mammococcus. The obtained results of examination and analysis of the experimental cheeses showed that the introduction of a large amount of pure Mammococcus culture into the pasteurized milk produced strong bitterness and a smearing consistency in every batch.

It is important to note that experimental cheeses of these batches differed from the control cheeses prepared from the same milk in their higher pH—6.03 (the control having pH 5.4), which indicated the low level of lactic acid fermentation.

The results of our investigations showed that the rate and direction of proteolysis were in fact different in the control and experimental cheeses.

The introduction of a large number of mammococci into the cheeses promoted an acceleration of the breakdown of protein to albumose and peptones.

At the age of two months the amount of peptone nitrogen in the experimental cheese was almost 1.6 times greater than in the control cheese.

The content of bitter-tasting amino acids in the total number of amino acids was 72.0% in the experimental cheese, and 63.9% in the control. Hence, the introduction of large numbers of mammococci into the cheese led to a change in the quantity of amino acids in the cheese. The more active accumulation of bitter-tasting amino acids, as compared with other amino acids, resulted in a deterioration of the taste owing to the production of strong bitterness.

The results of microbiological studies of the experimental cheeses showed that the numbers of mammococci increased considerably up to the age of one month, and then gradually fell as the cheese ripened.

A study of the features of the ripening of the cheese when 2% of pure Mammococcus culture was introduced into the pasteurized milk without the starter (variant IV) showed an even greater rate of protein breakdown and gave rise to strong and persistent bitterness and a smearing consistency.

SUMMARY

1. The degree of bitterness in ripening cheese depends largely on the numbers of bacteria of the Mammococcus group introduced into the milk.

2. The addition of different doses of pure Mammococcus culture to the milk simultaneously with the starter produces a change in the microfloral composition of the ripening cheese, persistent bitterness and smearing consistency, accelerates proteolysis, and changes the direction of this process towards increase in the quantity of primary protein breakdown products—peptones and some amino acids.

3. In cheeses containing Mammococcus the increase in the total free amino acids is accompanied by a change in their quantitative relationship towards the accumulation of bitter-tasting amino acids such as arginine, tryptophan, lysine, aspartic acid, valine, methionine, phenylalanine, leucine, and isoleucine.

4. In ripening cheese containing only Mammococcus culture there is a particularly accelerated rate of protein breakdown and this is accompanied by strong and persistent bitterness and smearing consistency.

5. A study of the bitter substances isolated from cheeses showed that they consisted of polypeptides and bitter amino acids.

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EASUREMENT OF rH_2 IN DEVELOPING BACTERIAL CULTURES BY MEANS OF CELL CONSISTING OF A PLATINUM AND ANTIMONY ELECTRODE

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In a number of works published during the last few ars reference has been made to the exceptional portance of research on the role of oxidation-rection process in the vital activity of microorganisms abotnova [1957]; Kramli [1956]). In particular, it is noted that investigation of the variation of the idation-reduction potential in developing bacterial cultures was a method which helped to reveal intricate biochemical processes.

An oxidation-reduction potential (ORP) arises on a platinum electrode immersed in the medium and expresses the ratio of oxidants and reductants in it.

The ORP is denoted by the symbol Eh and is meas-

Table for rH_2 Determination With a Cell Consisting of a Platinum and Antimony Electrode

rH_2	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	−250.0	−247.2	−244.3	−241.5	−238.6	−235.8	−233.0	−230.1	−227.3	−224.4
1	−221.6	−218.8	−215.9	−213.1	−210.2	−207.4	−204.6	−202.7	−199.9	−197.0
2	−194.2	−191.4	−188.5	−185.7	−182.8	−180.0	−177.5	−174.3	−171.5	−168.6
3	−165.8	−163.0	−160.1	−157.3	−154.4	−151.6	−148.8	−145.9	−143.1	−140.2
4	−137.4	−134.6	−131.7	−128.9	−126.0	−123.2	−120.4	−117.5	−114.7	−111.8
5	−109.0	−106.2	−103.3	−100.5	− 97.6	− 94.8	− 92.0	− 89.1	− 86.3	− 83.4
6	− 80.6	− 77.8	− 74.9	− 72.0	− 69.2	− 66.4	− 63.6	− 60.7	− 57.9	− 55.0
7	− 52.2	− 48.4	− 46.5	− 43.6	− 40.8	− 38.0	− 35.2	− 32.3	− 29.5	− 26.6
8	− 23.8	− 21.0	− 18.1	− 15.3	− 12.4	− 9.6	− 6.8	− 3.9	− 1.1	− 1.7
9	4.6	7.5	10.3	13.2	16.9	18.8	21.6	24.5	27.4	30.1
10	33.0	35.8	38.7	41.5	44.4	47.2	50.0	52.9	55.7	58.6
11	61.4	64.2	67.1	69.9	72.8	75.6	78.4	81.3	84.1	87.0
12	89.8	92.6	95.5	98.3	101.2	104.0	106.8	109.7	112.5	115.4
13	118.2	121.0	123.9	126.7	129.6	132.4	135.2	138.1	140.9	143.8
14	146.6	149.4	152.3	155.1	158.0	160.8	163.6	166.5	169.3	172.2
15	175.0	177.8	180.7	183.5	186.4	189.2	192.0	194.9	197.7	200.6
16	203.4	206.2	209.1	211.9	214.8	217.6	220.4	223.3	226.1	229.0
17	231.8	234.6	237.5	240.3	243.2	246.0	248.8	251.7	254.5	257.4
18	260.2	263.0	265.9	268.7	271.6	274.4	277.2	280.1	282.9	285.8
19	288.6	291.4	294.3	297.1	300.0	302.8	305.6	308.5	311.3	314.2
20	317.0	319.8	322.7	325.5	328.4	331.2	334.0	336.9	339.7	342.6
21	345.4	348.2	352.1	353.9	356.8	359.6	362.4	365.3	368.1	371.0
22	373.8	376.6	379.5	382.3	385.2	388.0	390.8	393.7	396.5	399.4
23	402.2	405.0	407.9	410.7	413.6	416.4	419.2	422.1	424.9	427.8
24	430.6	433.4	436.3	439.1	442.0	444.8	447.6	450.5	453.3	456.2
25	459.0	461.8	464.7	467.5	470.4	473.2	476.0	478.9	481.7	484.6
26	487.4	490.2	493.1	495.9	498.8	501.6	504.4	507.3	510.1	513.0
27	515.8	518.6	521.5	524.3	527.2	530.0	532.8	535.7	538.5	541.4
28	544.2	547.0	549.9	552.7	555.6	558.4	562.2	564.1	566.9	569.8
29	572.6	575.4	578.3	581.1	584.0	586.8	588.6	591.5	594.3	597.2
30	600.0	602.8	605.7	608.6	611.4	614.2	617.0	619.9	622.7	625.6
31	628.4	631.2	634.1	636.9	639.8	642.6	645.4	648.3	651.1	654.0
32	656.8	659.6	662.5	665.3	668.2	671.0	673.8	676.7	679.5	682.4
33	685.2	688.0	690.9	693.7	696.6	699.4	702.2	705.1	707.9	710.8
34	713.8	716.6	719.5	722.3	725.2	728.0	730.8	733.7	736.5	739.4
35	742.2	745.0	747.9	750.7	753.5	756.4	759.2	762.1	764.9	767.8
36	770.6	773.4	776.3	779.1	782.0	784.8	787.5	790.5	793.3	796.2
37	799.0	801.8	804.7	807.5	810.4	813.2	816.0	818.9	821.7	824.6
38	827.5	830.3	833.2	836.0	838.9	841.7	844.6	847.6	850.4	853.7
39	856.1	858.9	861.8	864.6	867.5	870.3	873.1	876.0	878.8	881.1
40	884.5	887.4	890.2	893.0	895.9	898.7	901.5	904.4	907.2	910.3
41	912.9									

ured in millivolts with reference to a standard hydrogen electrode.

The value Eh depends on the hydrogen-ion concentration. Even when the oxidation-reduction state of the medium remains constant a change of pH involves a change in the value Eh. Hence, in order to characterize the oxidation-reduction state we have to consider simultaneously the Eh and pH of the medium.

This led to the introduction of the symbol rH_2, which indicates the ORP of the medium with due regard to the pH. It is calculated from the formula

$$rH_2 = \frac{Eh}{30} + 2pH$$

It is true that the rH_2 does not in all media and at all pH values express sufficiently accurately the true ratio of oxidants and reductants. However, as Rabotnova's studies [1957] showed, in developing bacterial cultures, where the variaton of the pH does not usually exceed a few units, the inaccuracy of the rH_2, values is not great and can practically be neglected. The use of the index rH_2 is also based on the fact that it is possible to determine rH_2 of a medium directly, without separate determinations of Eh and pH.

Vles and Dex [1949] proposed a bimetallic cell consisting of a platinum and antimony electrode, and by means of which they recorded potentials from −250 to + 900 mv in solutions of hyposulfite, hypochlorite, and other substances. This scale of potentials corresponds to an rH_2 scale from 0 (−250 mv) to 41 (+900 mv).

In the literature at our disposal we could not find any works on the direct determination of rH_2 in developing bacterial cultures with a cell consisting of a platinum and antimony electrode, and this led us to conduct the present study.

In carrying out the first experiments we found that the standard factory-made antimony electrodes, which have a large area of contact between the antimony and the medium, were unsuitable for this purpose since their presence in the nutrient medium had an inhibiting effect on the growth of the culture.

Hence, we constructed an electrode with a much smaller surface from a fine rod of chemically pure antimony 1.5-2.0 mm thick, mounted in the end of a glass tube with BF-2 cement. The platinum electrode was made from 0.5 mm thick platinum wire sealed into a glass tube.

The two electrodes mounted in the cotton wool plug of the flask containing the nutrient medium, and before each experiment the surface of the antimony electrode was lightly cleaned, and the platinum electrode was platinized for 1 min in a 10% solution of platinum chloride at 4 v and was polarized in 10% sulfuric acid solution. In addition, a length of glass tube sealed with cotton wool was mounted in the plug.

When set up in this way the flask containing the nutrient was sterilized in an autoclave in the usual manner, and then the inoculum of the bacterial culture under investigation was introduced through the above-mentioned tube with a bacteriologucal loop. The electrodes were connected by mercury contacts to leads which were led out from the incubator to a panel, where they were attached to two terminals of a rose. The connection of the electrodes to the potentiometer (LP-5) was effected by means of a two-pin plug inserted into the rose.

In this way, we mounted 16 pairs of leads. Each pair was connected to the electrodes in one flask. This permitted the rapid connection of the potentiometer to the electrodes of each of the 16 flask containing the growing bacterial cultures, without removing them from the incubator, thus avoiding mixing of the medium and the concomitant change of ORP, and also considerably simplifying and speeding up the measurements.

To simplify the conversion of the potentiometer readings to rH_2 values we calculated and compiled a

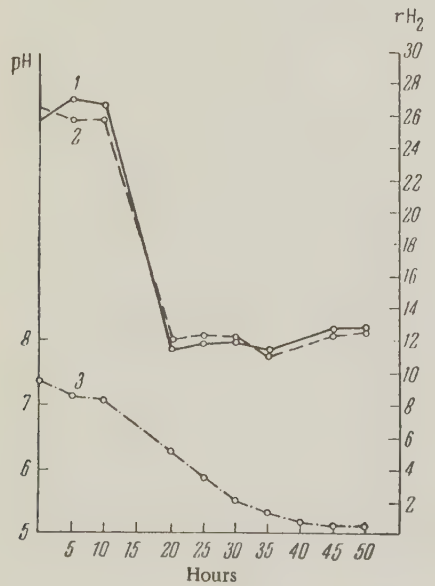


Fig. 1. rH_2 and pH in culture of Staphylococcus aureus in MPB. 1) rH_2 measured with platinum and antimony electrodes; 2) rH_2 measured with platinum and calomel electrodes; 3) pH.

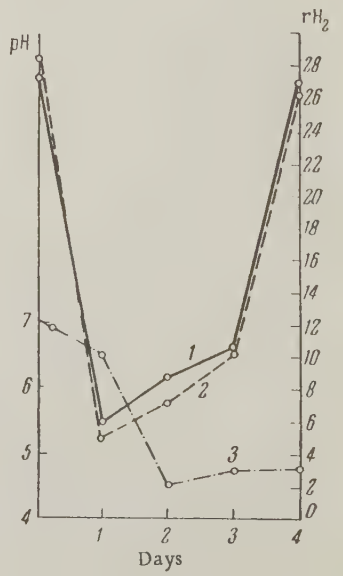


Fig. 2. rH_2 and pH in developing culture of B. coli in synthetic medium. Same designations.

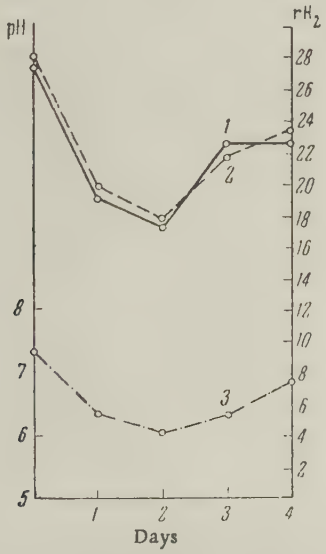
ecial table. The integral values of rH_2 are arranged the left-hand column, and the tenths in the upper w. The potentiometer reading in millivolts is sought the table, and from the values in the corresponding lumn and rows we can find the integer and tenths of e rH_2 value.

Example: potentiometer reading in millivolts=388, rresponding rH_2=22.5.

For potentiometer readings not given in the table e tenths of rH_2 are found by deducing the mean from jacent values.

In this way, we measured the rH_2 in cultures of coli, Staphylococcus aureus, S. citreus, B. pyocyeus, and B. prodigiosus, growing in MPB (pH=7.4) d also in a synthetic medium of the following compoion: distilled water—1 liter, glucose—2 g, sodium loride—5 g; ammonium sulfate—5 g, potassium phosate (disubstituted)—3 g, gelatin—3 g, nicotinic acid— mg (pH=7.0—7.4). The cultures were grown in 50 of medium at 37 deg.

All the measurements were accompanied by control terminations of the rH_2 by the conventional method a cell consisting of a platinum and saturated calomel ectrode. Here we used the same platinum electrode was introduced in pair with the antimony electrode, d the calomel electrode was connected to the culture rough a tube with asbestos fiber sealed into the bottom. The tube was filled with 10% KCl agar, mounted in the cotton wool plug of the flask, and sterilized along with the medium (Rabotnova's method [1957]).



. 3. rH_2 and pH in a developing culture of B. pyocyaneus in synthetic medium. Same designations.

The investigations showed that in the same developing bacterial cultures the rH_2 values, measured simultaneously by a cell consisting of a platinum and antimony electrode and the conventional method, practically coincided. Only in a few cases did we observe differences, which did not go beyond the limits of accuracy of the conventional method (1-2 rH_2 units). In repeated measurements of rH_2 during the entire development of the culture the results were the same. The curves plotted from the values determined by the two methods practically coincided, the deviations being within the limits of accuracy of the method (Figs. 1, 2, 3).

We conducted special experiments to find out if antimony electrodes of our suggested construction had any inhibiting effect on the growth and development of bacteria. It was found that when bacteria were cultured in liquid media with the antimony electrode present or absent the cultures formed suspensions of equal density, and when these were inoculated on to plates with MPA a similar number of uniform colonies grew. Cultures developing in the presence of an antimony electrode did not reduce the rH_2 of the medium quite as much as the controls, and this was particularly noticeable at low pH values (to 3-4 rH_2 units). This must be taken into account when the results of this direct determination of rH_2 are compared with the data of the usual method.

SUMMARY

1. By means of a cell consisting of a platinum and modified antimony electrode a direct measurement of rH_2 in developing bacterial cultures is possible.

2. In our proposed modification this method of determinining rH_2 in developing bacteria cultures is simpler and more convenient than the conventional one.

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CONTROL OF INOCULUM IN THE BIOLOGICAL OXIDATION OF SORBITOL TO SORBOSE

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The satisfactory conduction of biosynthetic processes in industry is governed by the following factors: 1) Quality of inoculum; 2) composition of culture media in which the biosynthesis takes place; 3) physical conditions, i.e., temperature, rate of mixing, amount of air supplied, etc.

Of the enumerated factors the most inconstant and variable is the quality of the inoculum, which depends on the number of microorganisms, their vitality and the age of the culture.

Any method of assessing these qualities would enable the biosynthesis to be carried out in the best conditions, thus leading to the obtention of reproducible results and to the avoidance of failures due to the inoculm. However, the methods employed for this purpose are either inadequate, or are long and inconvenient.

For instance, sometimes the method is restricted solely to a count of the number of microorganisms, it being assumed that the vitality and biosynthetic qualities of the culture are proportional to the number of cells, and this is only a very rough approximation.

The most reliable control of the whole biosynthetic process can be achieved by using part of the prepared inoculum for conducting biosynthesis in the laboratory, as is done, for instance, in penicillin production.

In the case of the industrial production of sorbose from sorbitol by means of the bacterium Acetobacter suboxydans this is impossible, since the inoculum deteriorates on keeping, and by the time the test of part of the inoculum is completed the bulk of it has lost its activity.

In a previous work we tried to find a method of controlling a culture of A. suboxydans used for the industrial production of sorbose.

It has been shown (Razumovskaya, 1956; Cummins et al. 1957) that A. suboxydans can oxidize sorbitol in the following ways:

1. Sorbitol → sorbose
2. Sorbitol → fructose
3. Sorbitol → hexose

The first two pathways are best investigated with the aid of isolated and purified preparations of the corresponding enzymes. It has been demonstrated that these two processes are effected by means of dehydrogenase. The conversion of sorbitol to sorbose can be represented in the following scheme:

$$\text{Sorbitol} + \text{TPN} \rightleftarrows \text{sorbose} - \text{TPN} : H_2$$
$$\text{TPN} \cdot H_2 \ldots\ldots + O_2 \rightleftarrows \text{TPN} + H_2O_2$$

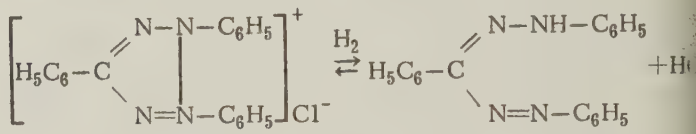
In the first stage sorbitol gives up two hydrogen atoms and changes it into sorbose.

In the second stage the reduction of dehydrogenase transfers two hydrogen atoms to the oxygen of the air, probably by means of other transporters.

From a consideration of this mechanism of oxidation we attempted to solve the posed problem by measuring the second stage of the process, i.e., the dehydrogenase activity of the culture.

For determining the dehydrogenase activity we used triphenyltetrazolium chloride. Triphenyltetrazolium chloride (TTZ) and its other derivatives are the best indicators of biological oxidation-reduction processes, since they enable quantitative photocolorimetric determinations.

When TTZ is used, triphenylformazan of red color is obtained.



Colorless TTZ — Red TPF

EXPERIMENTAL

The experiments were performed in conical 300 ml flasks each containing 50 ml of nutrient medium of the following composition (in %): technical sorbitol—15; maize extract—0.3; $CaCO_3$—0.5.

The flasks were inoculated with a pure culture of A. suboxydans and incubated on a shaker for 48 hr at 28 deg. The culture grown in these conditions was used as inoculum after its dehydrogenase activity had been determined with TTZ. In the first stage of our work the experiments on sorbitol-sorbose conversion were carried out in 300 ml glass flasks filled with the same medium and in the same conditions as in the preparation of the inoculum. In the second stage of the work 40 liter metal fermenters were used for the sorbitol oxidation. Air at pressure 2 atm was supplied at a rate of 0.5 liter/min per liter of medium; after 24 hours the sorbose content was determined by Bertrand's method.

DETERMINATION OF DEHYDROGENASE ACTIVITY OF CULTURE

Reagents:
1. TTZ—0.1% aqueous solution
2. Sorbitol—15% aqueous solution
3. Triphenylformazan, acetone solution—10 μ g/ml
4. Acetone

The inoculum was thoroughly shaken in order to give a uniform cell suspension, after which 4 ml was removed from it with a sterile pipet and put into a graduated centrifuge tube. It was centrifuged for 15 min at 3000 rpm. The liquid was removed and 1 ml each of TTZ solution and sorbitol was added to the remaining mass.

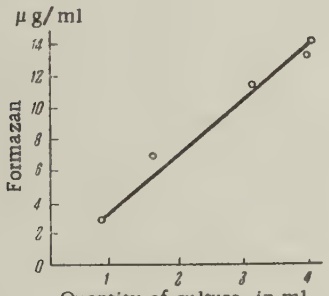
The mixture was mixed with a glass rod and incubated for 30 min at 37 deg, after which it was cen-

Table 1. Relation Between Degree of Sorbitol Oxidation in Fermenters and Quantity of Triphenylformazan Obtained in Test of Inoculum

o. of expt.	Triphenylformazan, μg/ml	Quantity of sorbose after 24 hr as % of sorbitol
1	0	0
2	0	0
3	2	0
4	6.7	43
5	8	48.7
6	10	80
7	10	83
8	13	96
9	13	89

Table 2. Relation Between Degree of Sorbitol Oxidation in Flasks on Shaker and Quantity of Triphenylformazan Obtained in Test of Inoculum

o. of expt.	Triphenylformazan, μg/ml	Quantity of sorbose after 24 hr as % of sorbitol
1	0	0
2	0	0
3	5.15	33.9
4	6	30
5	6	32
6	6.7	38
7	10	46
8	10.2	50
9	12	55
10	12	55
10	12.4	57



Relation between reduced triphenyltetrazolium chloride and biomass of A. suboxydans.

ifuged for 15 min at 3000 rpm. The clear liquid was moved. Four ml of acetone was added to the red sidue, and then the tube was heated in a boiling ıter bath for 30 sec. The tube was stoppered and thoroughly shaken. When cooled, it was centrifuged for 5 min. The volume was made up with pure acetone and the quantity of red formazan was determined by means of a standard solution (10 μ g/ml) and colorimeter or photocolorimetric curve.

RESULTS

1. Relation between size of microbial biomass and quantity of reduced TTZ.

Different volumes of the same inoculum were treated with TTZ by the method described above. The data obtained are shown in the figure. It can be seen that the amount of reduced TTZ is proportional to the quantity of microbial mass.

To verify the relationship between the viability and the reduction of TTZ we carried out experiments with heat-killed bacteria. After 30 min boiling of the culture no reduction of TTZ was observed.

2. Relation between degree of oxidation of sorbitol to sorbose and quantity of triphenylformazan obtained.

Tables 1 and 2 show the results of experiments conducted in metal fermenters and in glass flasks on a shaker.

In these experiments no sorbose could be detected after 24 hr by Bertrand's method in cases where no more than 2 μ g/ml of formazan was formed in the inoculum.

When an inoculum giving 5.15-6.7 μ g/ml of formazan was used, the yield of sorbose in glass flasks was 30-38. When the quantity of formazan was 10 μ g/ml or higher, the greater yields of sorbose in the given conditions were obtained.

Thus, by determining the quantity of formazan obtained from the reduction of TTZ we can select the best inoculum for individual fermentations and then strive to find congenial conditions for its preparation.

SUMMARY

1. A method is proposed for assaying the activity of the inoculum used for oxidation of sorbitol to sorbose in industrial conditions.

2. The method is based on the colorimetric determination of the quantity of red formazan formed from colorless tetrazolium acted on by Acetobacter suboxydans. The quantity of formazan is proportional to the vitality, oxidative activity, and density of the microbial population used for inoculation.

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BOOK REVIEWS

The Chemistry and Biology of Yeasts, edited by A. N. Cook
Academic Press Inc., New York, 1958, 763 pp.

A consequence of the exceptionally rapid and ever increasing differentiation of sciences which is taking place before our eyes is that individual scientists are generally no longer able to keep up directly with all the published material, not only in their own science as a whole, but frequently in its major subdivisions. Hence, the literature reviews now being published, even on these subdivisions, are usually complied by a group of investigators who are working on special problems.

Such a collective work is the book on yeasts which we review now.

Eminent specialists in microbiology and biochemistry from the U.S.A., England, Holland, Denmark, and Scotland have collaborated in its compilation.

The book contains 13 chapters, each of which is devoted to a particular problem in the study of yeasts.

The following are the titles of the chapters and the names of their authors.

I.* The Classification of Yeasts—J. Lodder, W. Ch. Slooff, and N. J. W. Kreger-van Rij.
II.* Ecology of Yeasts—A. Lund.
III.* Life History and Cytology of Yeasts—O. Winge and C. Roberts.
IV.* Yeast Genetics—O. Winge and C. Roberts.
V. Aspects of the Chemical Composition of Yeasts—A. A. Eddy.
VI. Yeast Growth—E. O. Morris.
VII. Fermentation and Respiration—F. F. Nord and S. Weiss.
VIII. Synthesis and Degradation of Cellular Carbohydrates by Yeasts—W. E. Trevelyan.
IX. Nitrogen Metabolism—G. Harris.
X.* The Technology of Yeast—M. Pyke.
XI.* Pathogenic Yeasts—G. C. Ainsworth.
XII.* Yeasts in Food Spoilage—M. Ingram.
XIII.* Flocculation of Yeasts—H. E. Jansen.

For greater speed and completeness in reviewing this voluminous work it was deemed advisable to share it between a biologist and biochemist.

This review will deal only with general biological questions and the material on yeast technology from the 8 chapters marked with an asterisk.

An acquaintance with the contents of all the chapters shows that their authors have taken into account almost all the important published results, especially of the last 10-15 years. The only exception is the results of Soviet investigators. This definitely reflects on the comprehensiveness and objectivity of the account of the significant trends and tendencies in the development of present-day microbiology, these qualities, according to the preface, being the main aims of the editor of the book.

The first chapter (pp. 1-62) is a short account of the book of the well-known Dutch taxonomists, Lodder and Kreger-van Rij, on yeast classification, and a review of some relevant new information published in the foreign press after its appearance. This latter information, however, has not affected the method and scheme of the former classification. Hence the critical observations which I made in a review of this book in 1955, still hold good (see Mikrobiologiya 24, 2 247-251).

As regards new information, the most interesting points are the following, which have still not found expression in our own publications.

In 1943, the Lindegrens reported that among laboratory asporogenous cultures of Sacch. cerevisiae which they had isolated from single spores, there were some which were of different mating types, and which on being mixed produced asci with spores, i.e., they recovered their ability for spore-formation.

Eight years later, Wickerham (1951), then Wickerham and Burton (1954), found a similar manifestation of heterothallism among cultures of yeasts isolated from nature and belonging to different species of asporogenous yeasts.

For instance, they found that if certain strains of Candida guilliermondii were mixed with suitable strains (of opposite mating type) of C. melibiosi, then conjugation occurred between their cells with the subsequent formation of hat-shaped spores of the Hansenula type.

The authors of the reviewed chapter are perfectly correct in noting that the use of this "mating" method of studying yeasts is a new way of finding out their true affinity. However, they still do not draw any practical conclusions as regards the transference of the named species to the genus Hansenula.

Of the newly described species particular interest attaches to S. diastaticus (Andrews and Gilliland, 1952). These yeasts were isolated from hyperfermented beer wort and were capable of using complex dextrins and even starch.

There are numerous other references to journal articles containing new information on yeasts. Some of these, contained in little-known journals with small circulations, have been unkown to a wide circle of microbiologists before this book appeared.

The bibliography contains 195 references.

The second chapter, "Ecology of Yeasts" (pp. 63-91), is written by the Danish microbiologist Lund. He himself has carried out extensive studies on the distribution of yeasts in nature, and these studies were summarized in a book entitled "Studies on the Ecology of Yeasts" (1954).

The reviewed volume gives a short account of the contents of this book, supplemented with new material published in the period up to the end of 1956 (there are about 150 literature references).

In the introduction the author notes that the isolation of yeasts from the original substrates has been greatly facilitated in recent years by the addition to the nutrient media of substances inhibiting the growth of other organisms, particularly molds. These include: diphenyl in amount 0.01%, sodium propionate in amounts 0.25-0.35%, and others.

This chapter gives a full list of species of yeasts found on dead and decomposing leaves, in the nectar of flowers, on fruits, cereal grains, root crops, in soil, dung, exudates of trees, insects, on fruiting bodies of fungi, and so on, and then in "industrial products"—beer, wine, cider, honey, syrup, milk and milk products, brines, wood pulp for the paper industry, etc.

This arrangement of organisms according to the substrates on which they are found is, in fact, a typical feature of ecology as the science of the interrelationships of living creatures with their habitat. However, this is only the first step of the ecologists, and must be followed by investigation and account of what factors determine the existence of particular organisms in each particular environment, i.e., their specific adaptations. The practical scientific value of this ecological information is obvious. It provides the biological industry, agriculture and medicine with the knowledge which will enable them to make the most effective use of beneficial microorganisms and to take the best measures against noxious ones. Unfortunately, the author deals with hardly any of these questions. Hence, the whole chapter is more an introduction to the ecology of yeasts. At the same time there is such information, and fairly fully too (excluding the results of Soviet investigators), in Chapter XII of the reviewed volume, under the title "Yeasts in Food Spoilage" (pp. 603-633). It is written by the English microbiologist M. Ingram, the author of the very valuable book "An Introduction to the Biology of Yeasts" 1954) (cf. Kudryavtsev. New Books From Abroad. Ser. B. No. 12, p. 38, 1956).

The object of this chapter, writes M. Ingram, is more to indicate how the biological properties of the yeasts determine their participation in food spoilage rather than to catalog the different kinds of yeasts concerned therein. For this purpose he describes the physiochemical characteristics of, for instance, cereal grains, fruits and fruit juices, sugar, honey, syrup, pickles and brines, meat and dairy products, etc., and also the specific properties of microorganisms, mainly yeasts, which enable them to develop in these products and cause their spoilage.

In the majority of cases the information given makes it quite apparent what favors and what limits the growth of particular microorganisms, and what measures should be taken to prevent their growth. In some cases reference is made also to the effect of artificial factors, antibiotics for instance, on microorganisms. Where no information is available on these points the author outlines interesting methods of further stufy.

Ingram's review is of great value for a wide circle of microbiologists, and especially for those working in the food industry.

There is a bibliography of 66 references.

In the short chapter, "Pathogenic Yeasts" (Chap. XI, pp. 587-602), the English mycologist Ainsworth had apparently the same aim—to give an ecological account of another special group of yeasts.

Here the author summarizes newly published information, mainly of the last decade, on Pityrosporum ovale, Candida albicans, Cryptococcus neoformans, on some dimorphic fungi—Blastomyces, Sporotrichum, etc., causing disease in man and higher animals, and also on yeasts pathogenic for lower animals and plants.

Unfortunately, our knowledge of what specific properties determine their pathogenicity is still very inadequate. Hence, besides the usual and brief description of the morphology and physiology of the aforesaid organisms on laboratory media, the author gives notes on their external form in the parasitic and saprophytic states, on their distribution and epidemiology, their ability to infect different organs and tissues of man and animals, the results of special experiments on infecting laboratory animals, and the action of some therapeutic substances.

In view of the great interest in pigeons at the present time, the cited fact of the discovery of the pathogenic yeasts Cryptococcus neoformans in pigeon droppings (in 63 out of 111 examined samples) is of some interest. It is suggested that pigeon excrement is a congenial medium for the saprophytic development of these yeasts. The epidimiological significance of this discovery is still not clear, writes the author, but pigeons may very well be involved in the epidemiology of outbreaks of cryptococcal mastitis in cattle.

Such, in brief, is the content of Chapters II, XI and XII, dealing with questions of yeast ecology.

Chapter III, "Life History and Cytology of Yeasts" (pp. 93-122) is written by the well-known Danish geneticists Winge and Roberts, who concentrate on the alternation of generations and mating types, sporulation, cytology, and polyploidy. Here the authors give a very schematic account of the available and, incidentally, still rather sparse information on the life histories of yeasts, and hence, arrive at certain questionable conclusions.

For instance, along with certain other authors which they cite, they continue to maintain that the previous distinction between the haploid genus Zygosaccheromyces and the diploid genus Saccheromyces is invalid. At the same time, these groups of organisms are completely different in their nature, and are easily distinguished in industrial practice. It is not at all accidental, for instance, that in the brewing, alcohol, and baking industries they use only yeasts with a developmental cycle typical of the genus Saccharomyces, and not of the genus Zygosaccharomyces.

Valuable information on sporulation in yeasts is given later. In particular, reference is made to certain constituents of the medium which promote or suppress this process.

In the following section, the authors point out that of all organisms yeasts are those which give rise to the greatest controversy as regards cytology, and the views on their nucleus and chromosomes are widely divergent. Hence, there is no direct evidence of ploidy in yeasts, but only indirect evidence, which is given in fair detail later on.

We cannot, however, fail to note the extreme controversy over the assessment of this evidence by different geneticists.

It is clear from this chapter that all the subjects dealt with have been the subject of extensive investigations abroad.

About 140 references are given in the literature list.

The following fourth chapter, "Yeast Genetics" (pp. 123-156) is written by the same authors as the third.

In the introduction it is noted that during the last 20 years yeast genetics have made remarkable rapid progress, and this is revealed in the studies of a great number of individual genes, in the construction of chromosome maps, in the demonstration of the existence of inbreeding degeneration, hybrid vigor, and so on.

All this is illustrated by numerous, though sometimes by rather unconvincing, examples.

As was to be expected, all the known facts of the experimental variability of organisms are explained by these authors, either by a combination of the existing genes or by mutational changes of these genes, irrespective of the changed conditions of existence.

From our viewpoint, such an approach to the solution of this problem is obviously one-sided, and that is why the authors cannot explain from the standpoint of genetics the mechanism, for instance, of the origin of hereditarily stable galactose-fermenting forms of yeasts in the process of their adaptation to galactose (long-term adaptation), after pointing out that the results of investigations of this process are contradictory.

Despite this one-sided approach, the geneticists have discovered some interesting facts. For instance, they refer to the existence in yeasts of genetically different enzymes of maltose and raffinose fermentation. In this connection it is noted that biochemical studies of enzymes in yeasts must now involve the participation of the geneticist, who can obtain yeasts containing only one particular type of maltose or raffinose, and not a mixture of them.

Mention is also made of the fact that artificial hybridization of industrial yeasts can be used to obtain forms possessing the best properties. However, appropriate examples are not given.

There is a bibliography of 120 references.

The tenth chapter, "The Technology of Yeast" (pp. 535-586) written by Magnus Pyke, is a good supplement to White's book "Yeast Technology" (1954), translated into Russian in 1957.

For instance, in the section "Dried Baker's Yeast" the author cites the interesting data of Pollock and Holmstrom (1951), which were apparently unknown to White at that time, from which it is seen that the productive activity of dried yeast at 21 deg is retained for two years only if they contain about 16% trehalose and 40% of total carbohydrate. With smaller amounts of these substances the dried yeast is unsatisfactory and unstable. Mention is also made of the fact that the trehalose content of yeasts can be increased if they are cultured in a nitrogen-deficient medium.

Some other observations and theories, more up-to-date than White's information, are given on the questions discussed. In particular, Pyke's review gives a fuller account of the food value of yeasts and of their use in medicine for prophylactic and therapeutic purposes. For instance, there are fairly full descriptions of recipes for preparing yeasts extracts and bouillons, and also the official specifications for the vitamin content of dried yeast, according to the American, English, and Swedish Pharmacopeias.

A special section of the review is devoted to questions of enrichment of yeast in vitamins B_1 and B_6, and in ergosterol for synthesis of vitamin D_2 and cortisone.

Noteworthy among the other interesting, newly published material is a possible, third method of obtaining glycerol by means of cetain osmophilic yeasts. According to investigations conducted in Canada (1955, 1956), these yeasts in aerated conditions produce substantial amounts of glycerol and also of D-arabitol.

In the thirteenth and last chapter, "Flocculation of Yeasts" (pp. 635-667), the Danish investigator Jansen first gives a fully annotated list of studies which attempted to ascribe flocculation mainly to the effect of particular constituents of the medium on the yeasts and of other conditions of their development. However, according to the author, these attempts did not solve the main question. The phenomenon is more complex, and is inexplicable by any single universal theory. The same conclusion is supported by research on the nature of the yeasts themselves, which has shown that in individual cases flocculation is a hereditary characteristic and hence is genetically controlled.

The author of this chapter considers these results of great value, although they do not fully explain the phenomenon in question. In order to obtain a better understanding of the mechanism of flocculation, he writes, we must also take into account factors depending on the surrounding medium, such as the electric charge of the cell wall, the adsorption and specific action of different substances, and so on, not only separately, but also in their mutual effect on yeasts. This obvious complication, he concludes, suggests that new fundamental approaches in the field of enzymology and cytology may prove of more general and ultimate value.

The bibliography contains 92 references.

In conclusion, it should be noted that all the chapters of the reviewed book are of great interest for a wide circle of microbiologists. They give, in compact and accessible form, an account of the main trends and tendencies of foreign scientific work on yeast organisms, which are becoming of more and more interest to manufacturers and scientific research workers.

V. I. Kudryavtsev.

INTERNATIONAL SYMPOSIUM ON ANTIBIOTICS

The International Symposium on Antibiotics, held in Prague during the 18th-30th of May of this year, aroused considerable interest in the scientific community. Representatives of 23 countries participated in it.

The symposium opened with introductory addresses by M. Herold, President of the Organization Committee, Academician I. Malek, and the Deputy Minister of Health of the CSR, who, after cordially welcoming the delegates to the symposium, gave interesting review papers on the technological problems of obtaining antibiotics, the use of antibiotics in agriculture and the food industry (M. Herold), the investigations in progress in the CSR in the field of antibiotics research (Academician Malek), and on the importance of antibiotics in public health (Deputy Minister of Health of the CSR).

The work of the symposium was carried out in three sections: general, technological, and medical.

The delegation from the USSR took part in the work of all sections and its members gave a number of papers. Altogether, about 200 papers were heard at the symposium. The papers and discussions dealt with questions of the biosynthesis of antibiotics, the use of antibiotics in medicine, agriculture, and the food industry, and the obtention of new antibiotics and their production.

Of course, in a brief report, we can single out only what we consider as the most important questions discussed during the proceedings of the symposium.

A great deal of attention was given to questions of the aeration and mixing of cultures in the production of antibiotics in laboratory and factory.

In the papers of V. Matolova, M. Herold, J. Necasek, and M. Bervida an account was given of solution of oxygen in flasks on a circular shaker designed by the authors, and in which it was possible to change not only the number of revolutions per minute (120-340 rpm) but also the radius of the circle described (from 2 to 45 mm). The authors showed that the amount of O_2 dissolved was linearly dependent on the number of revolutions of the shaker in unit time. Differences in the radius of the described circle had no appreciable effect on the solution of O_2. A considerable increase in the solution of O_2 was noted when flasks with glass agitator were used.

In Prague Scientific Research Institue of Antibiotics, work has been carried out on the evaluation of different methods of aeration and mixing under industrial conditions. The papers of B. Sikyta, J. Zajicok, and M. Herold discussed in detail the effect of different designs of mixers on the rate of O_2 solution. The Czechoslovak scientists concluded that the best results for the same consumption of electrial energy were given by a mixer of the open turbine type.

An interesting paper on the problem of aeration and O_2 requirement of cultures of antibiotic-producing organisms was given by R. Donovick (USA), who stressed the importance of ensuring that the O_2 entering the flasks (fermentors) or other containers had access to the surface of the cells. In comparing the means for supplying O_2 to fungal hyphae and bacterial cells, the author put forward the suggestion that one of the causes of the more rapid rate of metabolism in bacteria was that the cells of the latter were more accessible for oxygen.

The general and technological sections heard a number of papers dealing with the biosynthesis of antibiotic substances and the elucidation of the relationship between different types of metabolism and the structure of the antibiotic molecule.

Herold and other authors recommended that in investigations of the effect of the medium on antibiotic production the economic effect should be considered not only in terms of units per 1 ml of medium, but with regard to the total volume of the fermenter as well.

In his paper, Herold noted that a very promising method from the economic viewpoint was continuous fermentation, which enables the culture to be kept in the desired physiological state.

Prolonged cultivation of actinomycetes (A. lavindulae) in a flowing medium was the subject of a paper by M. Bekhterev and I. Kolesnikov (USSR).

A considerable stimulation of streptomycin biosynthesis was reported by F. Hodinor and F. Ulrich (Czechoslovakia), who described how a streptomycin yield, amounting to 6,000 units per 130 hr on enriched media containing starch, glucose, maize extract, and soya flour, could be increased to 200% by the employment of certain stimulators, particularly 5 ethyl-5-(1-ethyl-butyl)-thiobarbituric acid.

Z. Hostolek and Herold reported a 50-90% stimulation of chlortetracycline biosynthesis in the presence of benzyl thiocyanate.

A question deserving attention is the effect of iron on the biosynthesis of various antibiotics. In several papers it was revealed that Fe^{+} in the usual fermentation conditions suppresses the biosynthesis of streptomycin, chlortetracycline, oxytetracycline, and erythromycin.

The inhibitive effect of Fe++could be removed by the addition of methylene blue (J. Gado, Hungary), or by the creation of a protective silicate coating on the walls of iron fermenters (J. Zajicok, Czechoslovakia).

Of great interest were the studies on the effect of phosphorus on the biosynthesis of antibiotics. In several papers it was shown that inorganic phosphorus can play a decisive role in the very important biosyntheses of chlortetracyline, oxytetracycline, etc.

J. Doskocil (Czechoslovakia) found that during the development of A. aureofaciens the initial phosphorus content of the medium determined the total quantity of synthesized nucleic acids and the maximum respiration of the culture.

The production of chlortetracycline by A. aureofaciens begins after the inorganic phosphorus is used up, when the synthesis of nucleic acids ceases and the pyruvate concentration falls. An increased amount of phosphate has an adverse effect on chlortetracycline synthesis, especially when the maximum respiration becomes equal to the rate of oxygen solution.

N. Orlova and co-authors (USSR) showed that dissolved inorganic phosphorus is rapidly mobilized by the oxytetracycline producer and determines the rate of growth, the utilization and direction of metabolism of carbohydrates, the nature of the development of the producer, and the rate of oxytetracycline biosynthesis. An increase of the inorganic phosphorus content of the medium beyond the optimum point alters the course of all these processes, results in suppression of biosynthesis of the antibiotic, and also in an accumulation of a great quantity of organic acids, particularly acetic and pyruvic, in the medium.

The effect of phosphates on the nucleic acid metabolism of A. rimosus was discussed by T. Sik, J. Gado, and J. Horvoth (Hungary).

The biosynthesis of nystatin is appreciably affected by the concentration of inorganic phosphorus in the medium (L. Popova et al., USSR).

Great interest was aroused at the symposium by papers in which the authors tried to reveal the mechanism of formation of certain antibiotics. Since we are not able to dwell on these papers, we will merely point out that the great importance of organic acids in erythromycin synthesis was established.

The works of V. Musilek, and V. Vanek et al. (Czechoslovakia), showed that the biosynthesis of this antibiotic was associated with the decarboxylation of acetic acid. Using a synthetic medium, and studying the effect of salts of formic, propionic, pyruvic, acetic, and succinic acid on erythromycin biosynthesis, the authors concluded that the biosynthesis of propionic and formic acid proceeds from acetic acid and that these acids are intermediate products in the biosynthesis of the antibiotic, its lactonic ring.

In the case of oxytetracycline it was shown that stimulation of the biosynthesis of this antibiotic occurs when acetic and formic acids are present, and also in the presence of ethyl alcohol, which can be oxidized by catalase to acetic acid. The decisive role in the construction of the antibiotic molecule is attributed to acetic acid. In the biosynthesis of oxytetracycline an important role is played by flavin oxidases and oxidases containing Fe, particularly catalase. Interesting information on the mechanism of formation of polyacetylenic antibiotics and the relation between this process and the carbohydrate metabolism of the producers was given by J. D. BuLock (England).

R. Thomas (England) gave an account of studies to reveal the mechanism of alternariope biosynthesis. A paper by J. Kollar and M. Jarai (Hungary) dealt with the incorporation of chlorine into the chlortetracycline molecule.

There were a number of papers on investigations of the metabolism of antibiotic-producers and on the dynamics of growth (Z. Rchacek, Czechoslovakia; Severin et al., Egorov et al., Bezborodov et al., USSR).

The technological section heard six reports dealing with the breeding of antibiotic-producing microorganisms.

J. Darwas (Hungary) reported that multiple x-ray irradiation of strains of A. griseus, A. aureofaciens and A. rimosus resulted in the obtention of variants possessing activities 25-40% higher than that of the initial cultures. Variants with higher antibiotic productivity were distinguished by their greater ability to ferment sugar.

The paper of L. Erokhina (USSR) showed the effectiveness of the method of obtaining more active variants of the streptomycin producer by using multiple exposure to very high doses of ultraviolet rays with photoreactivation in the periods between exposures.

E. Paleckova (Czechoslovakia) compared the effectiveness of two mutagenic factors: ultraviolet rays and hydrochloride-methyl(dichlorodiethyl)-amine. The work was carried out with a culture of A. griseus strain LS-1. The author recommends the use of hydrochloride-methyl(dichlorodiethyl)-amine for repeated exposure, since the obtained strains, which are characterized by a high antibiotic productivity, do not show signs of degeneration, as is the case with repeated exposures to ultraviolet rays. The summaries of the papers of Prof. Sermonti (Italy) and Prof. L. Alikhanyan (USSR) refer to the great promise of using genetic methods (recombination and hybridization of antibiotic-producing microorganisms) for increasing the productivity of strains.

The papers dealing with the obtention of new antibiotics and their identification can be divided into 2 groups:

a) Papers dealing with general questions of method

b) Papers giving descriptions of the properties of antibiotics, methods of isolation, ways of chemical purification, etc.

A very interesting paper in the first group was that of D. Doskocilova (Czechoslovakia) on the use of infrared spectrophotometry for indentifying antibiotics in microquantities. She has devised a method of obtaining the IR spectra of substances by means of potassium bromide tablets containing about 0.2—1% of the antibiotic; the total quantity of antibiotic required for obtaining the tablet and determining the spectrum is only about 0.25 mg, which permits the identification of antibiotics in eluates from paper chromatograms. This method is of undoubted interest and deserves careful development.

Some questions of chemical methods of identification and study of new antibiotics were dealt with in the discussion on methods of investigating new antibiotics. In this discussion stress was laid on the prime importance of new physicochemical methods of studying antibiotics (chromatographic methods, spectrophotometry, etc.) for the most rapid investigation of new substances. At the same time, it was noted that accurate identification and investigation of the properties of an antibiotic is only possible after it has been isolated in large quantities.

The second group of papers, dealing with new antibiotics, was more numerous and the main attention

as devoted to antifungal, particularly polyenic, antiotics.

Information was given about several new antibiotics: ruscomycin (F. Marini, V. Panella, Italy); a new entoenic antibiotic (R. Brown, E. L. Hazen, U.S.A.); rubilin (J. Uri, J. Szilagyi, J. Bekesi, Hungary); bofugin (A. Khokhlov and Liberman, N. Solov'eva al., and others, USSR); an antifungal antibiotic milar to trichothecin (E. Glaz et al., Hungary); elanosporin and elaiphilin (F. Arcamone et al., aly); edeine (Z. Kurylo-Borowska, Poland), and hers. However, it is still impossible to speak with ıy great confidence about some of the described ıtibiotics as new ones (grubilin, particularly).

In the paper of D. A. Karnofsky (U.S.A.) on the use antitumor substances in clinical practice the forulas of the compounds used were given. They inuded the still unpublished formula of the antibiotic migallin (hypothetical) and of the antibiotic E-73. he last substance is very similar in structure to the ell-studied antibiotic actidione, and differs from it ıly in the presence of an additional acetoxy group.

The technological section of the symposium heard ıpers on the nonmedical use of antibiotic substances. These papers dealt with questions of the use of ıtibiotics for stimulating the growth and development farm animals, the use of antibiotics for combating seases and the possibility of employing antibiotic ıbstances for preserving products in fresh condition.

Particular attention was given to the employment of ıtibiotics in animal husbandry. All the speakers not-l the great economic effect of using antibiotics as owth stimulators for animals. The speakers demonrated the effectiveness of using antibiotics-enriched odstuffs for poultry, pigs, and calves.

The experience of Czechoslovakian specialists in ıe enrichment of fodders with antibiotics and vitamins ıdicated the immense possibilities for increasing the roductivity of animal husbandry by the employment these substances.

In papers read at the symposium it was noted that ıe use of antibiotics in animal feeding raises the esistance of the organism to various diseases, shock ıd radiation sickness, and improves the general ealth of the animals.

Along with the above-mentioned positive aspects, ıe speakers pointed out that attention must be given the appearance of antibiotic-resistant forms of athogenic microorganisms, to the allergic reactions the organism when these substances are used, and to the need for improving methods of revealing residues of antibiotics in the meat after the animals or birds have been slaughtered (M. Herold, Z. Muller, V. Lautner; J. Redl; Z. Zenisek; J. Bruggemann; Sarkisov et al.).

Papers on the use of antibiotics in plant growing were heard with great interest.

The preparation phytostrept, containing streptomycin and terramycin, was found to be effective against bacterial diseases of cucumbers, tomatoes and barley. The preparations actidione and fungicidin were tested against pericosporiosis of hops, anthracnose of haricot beans and other diseases of crops. A new method of employing actidione and fungicidin in the form of an aerosol reduces the phytotoxicity of actidione and the consumption of the antibiotics (M. Stanek, Czechoslovakia).

In a paper dealing with the effect of antibiotics on nodule bacteria, J. Vintika and H. Vintikova (Czechoslovakia) demonstrated the stimulating effect of small doses of penicillin on many strains of nodule bacteria and their adaptation to large doses of penicillin. These strains in practice proved to be more effective both as regards nodule formation and nitrogen fixation.

J. Kohler (Germany) delivered a paper on the use of polyenic antibiotics for plant protection.

H. B. Hawley (England) gave an account of the use of the antibiotics botrycidin and nisin in the brewing and dairy industries. These antibiotics were used for prevention and treatment of infections of productive cultures.

The medical section papers and some of the technological section papers dealt with questions of the therapeutic application of antibiotics, including such questions as the resistance of microorganisms to particular substances, the prophylaxis and treatment of candidomycosis and the interrelationships of antibiotics and microorganisms.

The delegates to the symposium were given the opportunity of acquainting themselves with the activity of Czechoslovakian microbiological institutions and of some antibiotic industries.

In concluding this short account of the work of the Prague Symposium on Antibiotics we wish to mention the warm hospitality of Czechoslovakian scientists, a hospitality which the Soviet delegates experienced throughout their stay in Czechoslovakia.

G. K. Skryabin, I. V. Konova, and Yu. M. Khokhlova.

DEFENCE OF DISSERTATIONS

In the Section of Biological Sciences AN UkSSR in Kiev on March 6, 1959, A.S. Bondarenko defended his dissertation for the degree of Candidate of Biological Sciences on the theme: Obtention and Investigation of an Antibiotic from the Lichen Cornicularia steppae Sav.

From the lichen C. steppae Sav., which grows in the USSR, three antibiotics were isolated in crystalline form. The most active of them was called steppin. According to Asahina's classification of lichen substances, steppin belongs to the category of aliphatic substances, to the fatty acid group, which agrees with the classification of lichen substances of Hesse and Zopf. Of the described lichen substances, steppin is most closely related to L-protolichesterinic acid, but in some physicochemical properties and in its activity against several microbial cultures it differs somewhat from it.

Steppin is very stable in the dry crystalline state, but it rapidly loses its antibacterial activity in alkaline solutions. It is extremely active against gram-positive, but has no effect against gram-negative bacteria (with certain exceptions), and is moderately active against fungi. It can be classed among the most active lichen antibiotics.

The antibacterial activity of steppin depends very largely on the medium in which the investigation is conducted. For instance, in Hottinger bouillon, steppin is more active than in meat-peptone bouillon; in acid media its activity is higher than in alkaline media. The protein fluids of an organism in vitro reduce or almost completely suppress the antibacterial activity of steppin.

Steppin is moderately toxic. It is less toxic than other lichen antibiotics. Steppin does not cause any local toxic effects on repeated rubbing of it on to normal or scarified skin of rabbits.

In view of the fairly high activity of steppin in vitro against several pathogenic microorganisms and of its other properties, an investigation of the therapeutic properties of this preparation is recommended.

In the Timiryazev Moscow Agricultural Academy on June 22, 1959, N. A. Sandrak defended his dissertation for the degree of Candidate of Biological Sciences on the theme: Chemical Composition of Extracellular Nitrogenous Excretions of Azotobacter and Their Availability as a Nitrogen Source for Microorganisms and Higher Plants.

It was established that Azotobacter chrococcum during its life excretes into the surrounding medium some of the nitrogen fixed by it.

The amount of nitrogen excreted by Azotobacter into the medium comprises 9-15% of the total amount of atmospheric nitrogen fixed by it. The composition of the nitrogenous excretions and their amount vary with the age of the culture: In the excretions of a three-day old Azotobacter culture alanine was found, while excretions of older cultures were found to contain ammonia and organic forms of nitrogen, dissociated in the acid hydrolysis of amino acid. In view of this it was suggested that these compounds are the simplest peptides or compounds of acids with other groups of organic compounds (carbohydrates, for instance). The amount of of ammonia increases with the age of the culture, but before the exhaustion of the sugar in the medium ammonia nitrogen does not exceed 27% of the total nitrogen excreted by Azotobacter into the medium. Hence, it was assumed that ammonia is not a primary product of nitrogen fixation. In the opposite event its amount would be greater in the period of enhanced fixation of nitrogen by Azotobacter, i.e., in young cultures, where the sugar is still not used up.

It was shown that the nitrogenous excretions of Azotobacter are used as a nitrogen source by various microorganisms both in pure culture and in a natural population of microorganisms dwelling in soil. The nitrogen excreted by Azotobacter is most fully utilized by molds (40-70%), less so by actinomycetes (25-35%), and bacteria (27%).

The accessibility of nitrogenous excretions of Azotobacter, for representatives of different groups of microorganisms in pure and mixed cultures, lead to the conclusion that in soil conditions also these excretions can be used by soil microorganisms as a nitrogen source.

Some molds cultured jointly with Azotobacter in nitrogenless media used not only the nitrogen of excretions, but also utilized a substantial amount of the nitrogen of dying Azotobacter cells.

In these cultures Azotobacter continued to fix atmospheric nitrogen when energy material was present, and as a result the total nitrogen content of the culture increased.

It was found that the nitrogen of Azotobacter excretions was partially available also for higher plants both in sterile and nonsterile conditions but was not utilized so fully by them as by lower plants (in sterile conditions 8.4%, in nonsterile 15.6-31.3%). The small amount of nitrogenous excretions and their incomplete utilization by higher plants indicate that they are of no great importance in the nitrogen nutrition of higher plants.

The beneficial effect of Azotobacter on crop yield is not simply due to its nitrogenous excretions serving as a nitrogen source for the plants, but also to its excretions of growth substances and its accumulating (in the presence of energy sources) bound nitrogen in the soil. The latter becomes assessible to plants after the death of Azotobacter and the decomposition of the organic nitrogen compounds contained in its cells by other bacteria.

A filtrate of Azotobacter culture, in amounts 25 and ml per 100 ml of nutrient medium, stimulates the productivity of atmospheric nitrogen fixation by Azotobacter by 46-49%.

n the Institute of Microbiology AN SSSR on June 26, 59 two dissertations for the degree of Candidate of ological Sciences were defended.

L. A. Seliverstova defended her dissertation on the eme: The Effect of Ionizing Radiation on Vitamin nthesis by Microorganisms.

onizing radiations have a considerable effect on the osynthesis, assimilation and intracellular distribu- n of group B vitamins. Vitamins are more stable a cell complex than in aqueous solutions. The irra- tion of yeasts with x-rays in doses 60-90 kr greatly creases the excretion of thiamine, pantothenic acid, d pyridoxine from the cells into the medium. This dicates the partial breakdown of structures into ich these vitamins are incorporated, and also a ange in the sorption properties and permeabiltiy of e irradiated cells. The ability to accumulate panto- enic acid from nutrient media by irradiated cells of ccharomyces cerevisiae (strain A), and also the bio- nthesis of this vitamin by irradiated cells of Toru- osis utilis and the closest succeeding generations re reduced when the irradiation dose was increased.

e amount of free and loosely-bound pantothenic acid cells of an irradiated culture of S. cerevisiae (strain was greater than in the control, but the total content this vitamin was 25% lower on the average after ir- diation with doses of 60-90 kr.

Gamma irradiation with radioactive cobalt produced nsiderable changes in the riboflavin content of irra- ted, still-metabolizing yeast cells and their closest ogeny. In this case, the free and bound fractions of oflavin in irradiated cultures of S. cerevisiae (doses and 100 kr) were much smaller. At the same time, e ability of irradiated cells to accumulate riboflavin m the nutrient medium was greater than that of e control. T. utilis and Candida guilliermondii re- onded in a different way to irradiation. With increase the irradiation dose the content of free and bound oflavin increased.

onizing radiations caused a disturbance of the pyri- ine balance in yeast organisms. The amount of e and loosely bound pyridoxine in irradiated S. evisiae (strain A) cells and their nearest progeny 35% lower on the average than in nonirradiated ls. Similar changes in the free pyridoxine content e observed also in the auxotrophic organism T. is. In cells of irradiated yeasts substantial changes the para-aminobenzoic acid content were found. e amount of the latter in cells of the closest prog- of a S. cerevisiae culture irradiated with doses of 100 kr was 70% higher on the average, and in T. is 2-3 times higher, than in the control. Under the ion of 60-90 kr doses of x-rays, synthesis of the amin B_{12} complex in B. megaterium and Propioni- terium chermanii was greatly reduced. X-rays sed significant changes in the synthesis of meso- sitol in yeasts. The ability to accumulate meso- sitol in the closest progeny of irradiated S. cere- iae cells was greater than in the control, and in utilis it was many times greater.

No significant changes in thiamine synthesis in an radiated culture of A. cerevisiae (strain A) could be detected. There were no significant changes in the synthesis of nicotinic acid in S. cerevisiae and T. utilis irradiated with a dose of 100 kr.

A microbiological method was devised for determining para-aminobenzoic acid by using the yeast Rhodotorula aurantiaca, which is incapable of synthesizing this vitamin.

N. M. Gerasimova defended her dissertation for the degree of Candidate of Biological Sciences on the theme: "Features of the Carbohydrate Metabolism of Yeasts of the Genera Pichia and Hansenula."

It was shown that differences in the life histories of the genera Hansenula and Pichia were correlated with the different nature of carbohydrate metabolism in these microorganisms.

All the investigated sporogenous yeasts, classed in the genus Hansenula according to their developmental morphology, developed on liquid sugar-containing substrates by simultaneous fermentation and oxidation of sugars. In contrast to them, yeasts classed in the genus Pichia in view of their common morphology during development, did not caused fermentation of sugars, but developed exclusively by their oxidation. In development on synthetic medium containing glucose all species of yeasts of the genus Hansenula rapidly utilized sugar in the medium by fermentation and oxidation.

In this case, the following products of carbohydrate metabolism were formed: alcohol, carbon dioxide, volatile and nonvolatile acids, and volatile neutral esters. The nonvolatile acids found included citric, malic, lactic, succinic, and three undetermined acids.

As regards volatile acids, only acetic acid was found. The volatile neutral esters found included ethyl acetate. All these products, except carbon dioxide, were utilized by yeasts of the genus Hansenula for development when all the glucose in the medium had been used up. In certain specially active strains of H. anomala this process was very rapid and as a result there was a fairly considerable increase in the yeast biomass due to the utilization of metabolic products.

It was found that yeasts of the genus Pichia in the same conditions were much slower than yeasts of the genus Hansenula in using up the glucose in the medium and produced only carbon dioxide and nonvolatile acids. Identification of the latter revealed lactic and succinic acid.

When yeasts of the genera Hansenula and Pichia were grown on malt wort the same general pattern was noted in the formation and relative amounts of metabolic products, as was the case when they were grown on synthetic medium containing glucose.

It was found that the respiration rate of yeasts of the genus Hansenula was higher than that of Pichia. Besides this, the ratio of the CO_2 evolved to the O_2 absorbed in Hansenula was always greater than unity, while in Pichia it was less than unity.

Studies of these yeasts confirmed the classification principles according to which organisms forming hemispherical spores are assigned to the genus Pichia, and organisms forming hatlike spores with projecting

edges are assigned to the genus Hansenula. The determining feature is that this form of spore in the genus Pichia is always correlated with the ability to assimilate carbohydrates in the culture fluid only by oxidation, whereas in the genus Hansenula they can be assimilated simultaneously by oxidation and fermentation.

A. E. Kosmachev.

Guta Markovna Frenkel'

On August 23, 1959, Prof. Guta Markovna Frenkel', octor of Biological Sciences, Director of the Division Microbiology of Fermentative Processes of the D. K. abolotnyi Institute of Microbiology AN UkSSR, died ter a serious illness.

G. M. Frenkel' was born in 1896, in the town of ovgorod-Severskii, Chernigov region. On finishing e gymnasium in 1916, she entered the natural science ction of the Physics and Mathematics Department of ev Institute of Public Education. On completing her udies at the Institute she worked (1921-1931) as a cteriologist in the bacteriological laboratory of the entral Workers' Polyclinic in Kiev. By this time, ita Markovna had already started her scientific work. iring her service in the polyclinic she published 7 ientific works on bacteriology and serology, one of em being a monograph in collaboration with Prof. . G. Ben'yash.

From 1931, G. M. Frenkel' was employed as a senior ientific worker in the D. K. Zabolotnyi Institute of icrobiology AN UkSSR, and from 1936, she directed e Division of Anaerobic Microorganisms, which in 952 was combined with the Division of Industrial icrobiology and became the Division of Microbiology f Fermentative Processes. She directed this division ntil the last days of her life.

Over a period of several years she carried out research on the variability of microorganisms, and these tudies were summarized in the form of a dissertation n the theme "Dissociation of Bacteria," for which she as awarded the degree of Doctor of Biological ciences in 1953.

Guta Markovna Frenkel' is the author of 72 works ealing with bacteriophage, bacterial variability, anerobiosis, physiology of anaerobic, and fermentative icroorganisms. In 1956, her monograph Biology of Anaerobes and Anaerobiosis was published. She was the first to describe the whooping-cough bacteriophage and the Cl. sporogenes phage. She bred active strains of acetone-butyl bacteria and put forward the hypothesis of the conjugation of respiration and fermentation, a problem on which she worked during the last years of her life.

Guta Markovna combined her great scientific research work with teaching activities, and under her guidance 8 dissertations for the degree of Candidate of Sciences were completed and defended.

G. M. Frenkel' performed a great deal of scientific-social work: She was a member of the Academic Council AN UkSSR on the problem, Control of Metabolism of Microorganisms for the Purpose of Regulating Microbiological Processes in Industry and Agriculture, and was the leader of this work in the Institute of Microbiology AN UkSSR; she was also a member of the Academic Council of the Biological Section of the AN UkSSR, a member of the Academic Technical Council of the Kiev Soviet of National Economy, and a member of the editorial board of the Microbiological Journal AN UkSSR.

G. M. Frenkel' was awarded the medals "For Working Valour" and "For Valiant Work During the Great Patriotic War 1941-1945".

Guta Markovna was an alert, warm-hearted person, a good comrade, a friend of youth, and was always ready to share her experience and great knowledge.

A bright memory of her will remain forever in the hearts of all those who were closely acquainted with her.

P. E. Vizir'
V. G. Drobot'ko
M. K. Karpenko
I. D. Kolchinskaya
L. A. Liberman
V. V. Lipshits
L. Yu. Medvinskaya
L. I. Rubenchik
M. B. Tevilevich

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Ekspti. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

SIGNIFICANCE OF ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN SOVIET PERIODICALS

FIAN	Phys. Inst. Acad. Sci. USSR.
GDI	Water Power Inst.
GITI	State Sci.-Tech. Press
GITTL	State Tech. and Theor. Lit. Press
GONTI	State United Sci.-Tech. Press
Gosenergoizdat	State Power Press
Goskhimizdat	State Chem. Press
GOST	All-Union State Standard
GTTI	State Tech. and Theor. Lit. Press
IL	Foreign Lit. Press
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd. AN SSSR	Acad. Sci. USSR Press
Izd. MGU	Moscow State Univ. Press
LEIIZhT	Leningrad Power Inst. of Railroad Engineering
LET	Leningrad Elec. Engr. School
LETI	Leningrad Electrotechnical Inst.
LETIIZhT	Leningrad Electrical Engineering Research Inst. of Railroad Engr.
Mashgiz	State Sci.-Tech. Press for Machine Construction Lit.
MEP	Ministry of Electrical Industry
MES	Ministry of Electrical Power Plants
MESEP	Ministry of Electrical Power Plants and the Electrical Industry
MGU	Moscow State Univ.
MKhTI	Moscow Inst. Chem. Tech.
MOPI	Moscow Regional Pedagogical Inst.
MSP	Ministry of Industrial Construction
NII ZVUKSZAPIOI	Scientific Research Inst. of Sound Recording
NIKFI	Sci. Inst. of Modern Motion Picture Photography
ONTI	United Sci.-Tech. Press
OTI	Division of Technical Information
OTN	Div. Tech. Sci.
Stroiizdat	Construction Press
TOE	Association of Power Engineers
TsKTI	Central Research Inst. for Boilers and Turbines
TsNIEL	Central Scientific Research Elec. Engr. Lab.
TsNIEL-MES	Central Scientific Research Elec. Engr. Lab.–Ministry of Electric Power Plants
TsVTI	Central Office of Economic Information
UF	Ural Branch
VIESKh	All-Union Inst. of Rural Elec. Power Stations
VNIIM	All-Union Scientific Research Inst. of Meteorology
VNIIZhDT	All-Union Scientific Research Inst. of Railroad Engineering
VTI	All-Union Thermotech. Inst.
VZEI	All-Union Power Correspondence Inst.

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. – Publisher.

AIBS Russian Monograph Translations

The AIBS is in the process of expanding its Russian Translations Program extensively. Funds to subsidize translation and publication of important Russian literature in biology have been obtained from the National Science Foundation, as part of a larger program to encourage the exchange of scientific information between the two countries. The following monographs have been published:

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Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.
68 pp. $3.00, individuals and industrial libraries (U.S.A. and Canada);
$2.00, AIBS members and all other libraries; $.50, additional, foreign.

Essays on the Evolutionary Morphology of Plants. *By A. L. Takhtajan.*
Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.
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Problems in the Classification of Antagonists of Actinomycetes. *By G. F. Gause.*
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Arachnida. Vol. IV, No. 2. Fauna of the U.S.S.R. *By B. I. Pomerantzev.*
Edited by George Anastos. Translated by Alena Elbl.
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This is a translation of all papers in the Biological Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections in many areas of biology, among which are the following: biophysics, cytology, embryology, experimental morphology, genetics, histology, hydrobiology, microbiology, morphology, physiology, and zoology.

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(POCHVOVEDENIYE)

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